Impacts of Human Activities on Cold-Water Sponges and the Habitats They Form in the North East Atlantic

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Abstract

Sponges are filter-feeders present in many aquatic environments, from rivers to abyssal plains. In the deep-sea, sponges can form dense aggregations (sponge grounds) which constitute important but vulnerable ecosystems. As human activities now take place in the deep-sea, the need to determine the resilience of sponge grounds to disturbances is essential. My PhD aimed at understanding the impacts of oil and gas activities on deep-sea sponges and sponge grounds. First, a literature review on the known impacts of hydrocarbon production on sponge grounds is provided. Second, environmental data from industrial, academic and governmental sources were analysed to determine the impact of anthropogenic activities on sponge grounds. In a case study of the Faroe-Shetland channel, I showed that substrate characteristics and seawater temperature, modulated by anthropogenic activities, controlled the distribution of megafauna. Third, to determine the impacts of an oil spill on sponges, experiments exposing a shallow-water sponge model to crude oil and dispersant contaminated seawater or sediments were conducted. Through this experimental work, I showed that exposure to contaminants decreased the sponge filtering abilities. Furthermore, over 1000 genes, from the sponge and its associated microbes were detected as differentially expressed after exposure to contaminants. These effects were worsened by the addition of dispersant and their use within sponge grounds should therefore be avoided.

***footnote:** The view is better on top of the boulder. Thanks to Johanne for this comic idea!*

"Piled Higher and Deeper" by Jorge Cham

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1.1. Overview

This chapter details the current scientific knowledge on the potential impacts of offshore oil and gas activities on deep-sea sponges and was published as a literature review in Advances in Marine Biology (see appendix A).

1.2. Introduction

Presently, offshore oil and gas production accounts for one third of worldwide hydrocarbon production (Bennear, 2015). Since the end of the 1960s and the beginning of offshore oil and gas exploration, the oil and gas industry has developed technologies that enable exploitation of deep-sea environments (Managi *et al.*, 2005) and is, today, operating in deep and complex marine settings (Muehlenbachs *et al.*, 2013). Hydrocarbon exploration and production is taking place in areas where vulnerable benthic species such as deep-sea sponges are present. For example, in the Faroe-Shetland channel, oil production activities are taking place within a nature conservation marine protected area designated to protect the local deep-sea sponge grounds (Henry and Roberts, 2014).

Exploration for hydrocarbon and other resources in deep waters has helped discover new deep-sea environments. For example, collaborative efforts between academia and industry partners have been very successful in increasing our understanding of deep-sea benthic ecosystems, e.g., the SERPENT project (scientific and environmental remotely operated vehicle partnership using existing industrial technology) (Gates *et al.*, 2017) and discovering previously unknown habitats such as the Darwin Mounds in the NE Atlantic (Huvenne *et al.*, 2016). However, industrial operations in deeper settings are strongly correlated with recorded numbers of technical incidents such as blowouts, injuries or spills (Muehlenbachs *et al.*, 2013). This was most starkly demonstrated by the 2010 Deepwater Horizon oil spill in the Gulf of Mexico, caused by a well blowout at 1500 m depth (Beyer *et al.*, 2016). Subsea well blowouts and pipeline leaks at depth have become more of a concern, while the number of tanker-related oil incidents at surface has decreased over time (Jernelöv, 2010). In addition, day-to-day operations can also have environmental impacts in the deep sea (Cordes *et al.*, 2016). From the presence of man-made infrastructures on the seabed to the release of produced waters or the resedimentation of particles close to the drilling locations, the ecological footprints of the offshore oil and gas production activities are diverse (Kark *et al.*, 2015). As it is known that recovery rates vary in the deep sea depending on the region and biological communities already living there, understanding the impact of oil and gas industry-related activities on deep-sea benthic ecosystems is complex (Henry *et al.*, 2017).

While pressures from anthropogenic activities such as the exploitation of oil and gas reserves on deep-sea ecosystems keep increasing, our understanding of deep-sea organisms and the scale of human impacts on ecosystem functioning remains limited Deep-sea ecosystems comprise a highly diverse set of physical and biological settings, many of which are hotspots of biodiversity including abyssal plains, manganese nodule fields, cold-water coral reefs and sponge grounds (Ramirez-Llodra *et al.*, 2011). Although many of these ecosystems may contribute significantly in global biogeochemical cycling, the overall value of the ecosystem services provided by deep-sea ecosystems remains poorly quantified (Thurber *et al.*, 2014).

Sponges (phylum Porifera) play vital roles in sustaining global deep-sea biodiversity and ecosystem functioning. The diversity of sponges in the deep sea (Figure 1-1AB), the rarity of some poriferan taxa (members of the class Calcarea) and the ecological uniqueness of some poriferan groups such as carnivorous sponges of the family Cladorhizidae (Figure 1-1C) and the stalked glass sponges of the family Hyalonematidae all add to the biological richness of life in the deep ocean (Hogg *et al.*, 2010). Habitats formed by dense aggregations of one or several sponge taxa (sponge "grounds", Figure 1-1D) can extend over very large areas up to hundreds of km^2 and provide three-dimensionally complex and stable habitats that support distinct biological communities (Maldonado *et al.*, 2016). Maldonado *et al.* (2016) provide an extensive review of sponge grounds including deep-sea sponge grounds such as the hexactinellid sponge reefs in the North-East Pacific Ocean off British Colombia, astrophorid sponge aggregations in the North Atlantic, lithistid sponge grounds or Antarctic sponge grounds more than 400 species rich. Sponges themselves host an array of organisms ranging from bryozoans or polychaetes to crustaceans (Wulff, 2006; Kazanidis *et al.*, 2016). Sponge grounds act as nursery grounds and support many benthic species including commercially-important fish species such as rockfish, hake and blue ling (Freese and Wing, 2003; Du Preez and Tunnicliffe, 2011; Maldonado *et al.*, 2016) (Figure 1-1E–H). Therefore, sponge grounds meet several criteria of vulnerable marine ecosystems (VMEs) as recognised by the United Nations (UN) Food and Agriculture Organisation (FAO). Deep-sea sponge grounds also meet the criteria of ecologically or biologically significant areas (EBSAs) as defined by the UN Convention on Biological Diversity (Table 1-1) (Hogg *et al.*, 2010).

Despite their ability to enhance benthic biodiversity, the biology and ecology of deep-sea sponges has only just started to be uncovered. What has been revealed most

recently is that sponges play essential roles in the biogeochemical cycling of organic matter in the deep oceans (Cathalot *et al.*, 2015). This is principally owing to sponges being very efficient at filtering large volumes of water as they rely on particulate organic matter (POM) as well as dissolved organic matter (DOM) for food (Rix *et al.*, 2016). Up to 40% of the carbon and nitrogen assimilated by sponges are released back into the water column in the form of sponge detritus (Rix *et al.*, 2016). Sponges, including deep-sea species, thus recycle DOM to POM which is then available for other benthic organisms and contributes to the bentho-pelagic coupling in oligotrophic environments (Maldonado, 2016; Rix *et al.*, 2016). Sponges host highly diverse microbial communities of bacteria, archaea and eukaryotes, often compared for their complexity to the microbial assemblages of the mammalian gut (Hentschel *et al.*, 2012; Webster and Taylor, 2012). Evidence for the presence chemoautotrophic symbionts in deep-sea sponges has been found, with chemoautotrophic sponge holobionts contributing to primary productivity in deep-sea environments (Pita *et al.*, 2018). Deep-sea sponges participate also in nitrogen cycling through their microbial symbionts capable of nitrification, denitrification and anammox reactions (Hoffmann *et al.*, 2009; Li *et al.*, 2014). The concept of a "sponge loop" has therefore emerged in the literature whereby sponges support oligotrophic food webs by recycling organic as well as inorganic carbon and nitrogen (De Goeij *et al.*, 2013; Maldonado, 2016). Furthermore, sponge skeleton elements (spicules) are composed of silica assimilated from the environment and sponges play an important role in the cycling of silica. Glass sponge reefs composed of hexactinellid sponges such as *Aphrocallistes vastus*, which are composed of up to 80% of biogenic silica, concentrate huge amounts of Si in some areas of the seabed (Chu *et al.*, 2011).

It is also becoming more evident that deep-sea sponges create other ecosystem services: these "provisioning" services include the production of bioactive secondary metabolites related to sponge microbial associations that are of great interest to the biotechnology sector (Sipkema *et al.*, 2005; Jackson *et al.*, 2015). Conservation of these ancient animals (individual sponges have been aged over 400 to over 2000 years old) (McMurray *et al.*, 2008; Fallon *et al.*, 2010) and their habitats must therefore scale up with the rates and extent of emerging anthropogenic activities, and thus the impacts that deep-water oil and gas activities could have on these benthic organisms need to be considered in management plans.

The objective of this chapter is to provide a fully comprehensive review of the impacts of offshore oil and gas activities on deep-sea sponges and the habitats they create.

Although studies on the resilience of deep-sea sponges to some oil and gas production activities are starting to emerge, many knowledge gaps persist. Relevant findings from shallow-water sponges or other benthic organisms have therefore also been used here to highlight possible impacts on deep-sea sponges and deep-sea sponge grounds. The risks involved with this strategy will be discussed in the synthesis of the thesis available in chapter 5. Impacts can occur at all stages of offshore oil and gas activities from exploration, development and production through to decommissioning and legacy effects. Furthermore, effects of these activities can be detected across ecological scales from community, individual and cellular levels. This chapter therefore adopts a multiple-scale framework to assess impacts at the level of sponge habitats, at the individual sponge level and at the cellular and molecular level.

1.3. Effects on sponge habitats and communities

1.3.1. Impacts of routine activities on deep-sea sponge grounds

Subsea infrastructures (wells, pipelines, manifolds and platforms)

During the phases of exploration and development, offshore oil and gas activities require the drilling of wells and the installations of heavy infrastructure such as manifolds and pipelines that directly disturbs the seabed (Figure 1-2). Physical disruption and smothering by sediments is one of the main impacts linked to the early stages of oil field development arising from installing pipelines, cables, bottom rigs, templates, skids and platforms including platform legs and anchoring (OSPAR Commision, 2010). Physical disruption and increased sedimentation (Figure 1-1IJ) during these phases can locally diminish benthic communities by more than 90% in terms of megafaunal density within sponge grounds (Jones *et al.*, 2006). Long-term effects on deep-sea sponge grounds from such physical disturbance are still detectable up to 10 years post drilling and this slow, partial recovery, inversely related to the distance to the well and the time after drilling, could result from the long-lived nature, slow growth rates and low reproduction rates of most deep-sea organisms (Jones *et al.*, 2012). Very limited recovery of megafauna was observed in areas where drill cuttings were not eroded 10 years post-drilling (Jones *et al.*, 2012).

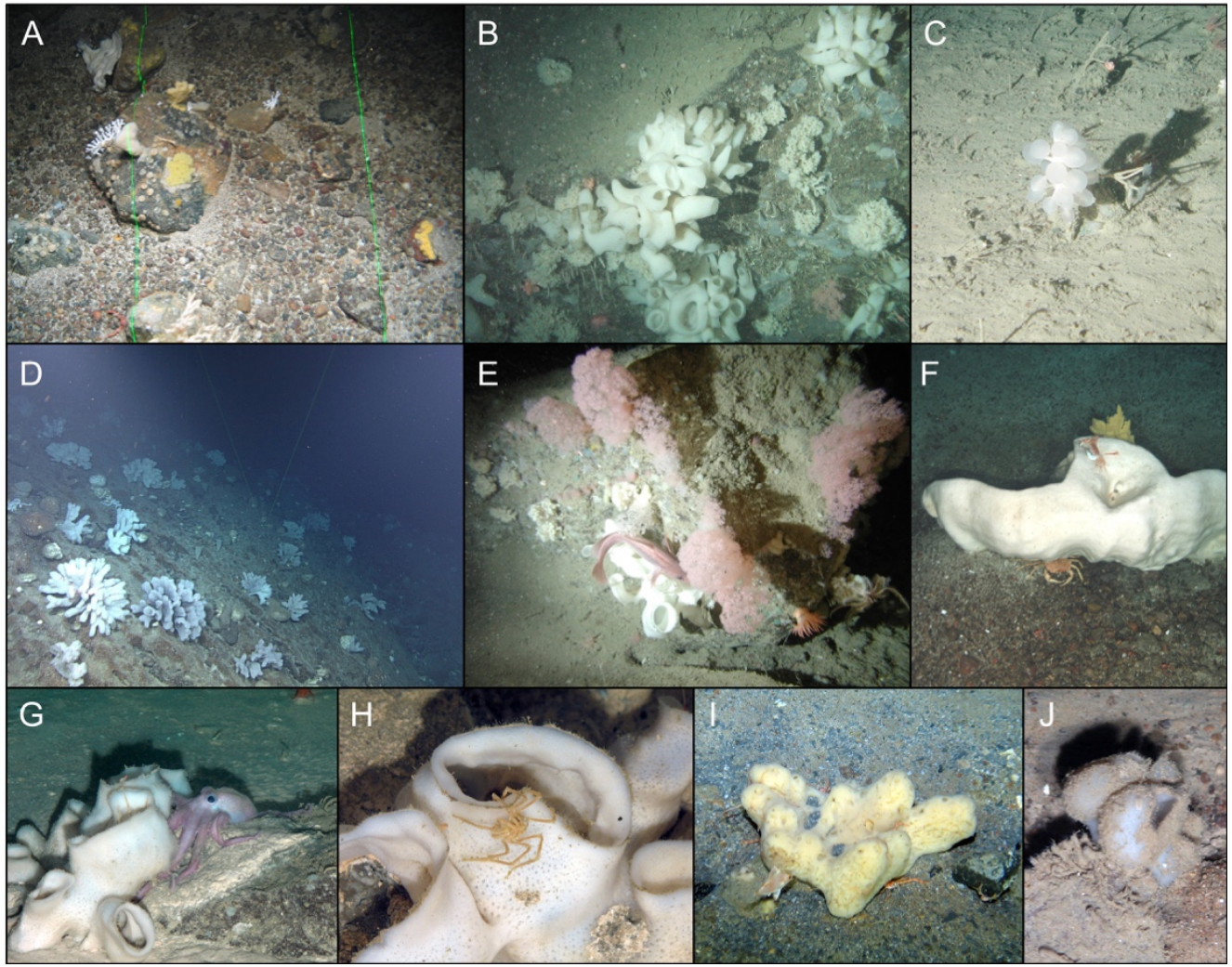

Figure 1-1. Examples of deep-sea sponges and of the habitats they form. (A, B) Example of deep-sea sponge morphotypes from the Faroe-Shetland channel. (C) Carnivorous sponges of the family Cladhorizidae constitute a deep-sea ecological oddity. (D) Present in high abundances, deep-sea sponges can form sponge grounds as seen here at 1890m depth from the Orphan Knoll, NW Atlantic. (E to H) Deep-sea sponges and sponge grounds provide habitats for various benthic organisms. (I and J) Sponges are impacted by offshore oil and gas activities amongst other through increased sedimentation. Photo credits: (D) Fisheries & Oceans (DFO), Canada. (G to I) SERPENT Project, National Oceanography Centre, Southampton UK.

Table 1-1. Vulnerable marine ecosystem (VME) and ecologically and biologically significant area (EBSA) criteria and their applicability to sponge grounds. For clarity, key words are highlighted in bold font.

Designation	Criteria	Characteristics of deep-sea sponges and/or sponge grounds fulfilling criteria
VME	Uniqueness or rarity	Deep-sea sponge grounds are not rare but occur in **specific and limited areas** where favourable abiotic conditions are present
	Functional significance of habitats	Deep-sea sponges increase **physical heterogeneity** of benthic ecosystems
	Fragility	Deep-sea sponges are **extremely vulnerable to physical damage** by trawling or other anthropogenic activities
	Life history traits making recovery difficult	Deep-sea sponges are considered as **slow-growing, long lived** organisms and their reproduction cycles are largely unknown
	Structural complexity	Deep-sea sponge grounds give **three-dimensionality** to seabed increasing the number of available microhabitats
EBSA	Uniqueness or rarity	Deep-sea sponge grounds are not rare but occur in **specific and limited areas** where favourable abiotic conditions are present
	Special importance for life history stages of species	Deep-sea sponge grounds constitute **nursery grounds** for fish and invertebrate species
	Importance for threatened, endangered or declining species and/or habitats	Deep-sea sponge grounds constitute nursery grounds for **threaten species** such as economically important fishes
	Vulnerability, fragility, sensitivity or slow recovery	Deep-sea sponges are considered as **slow-growing, long-lived** organisms, making them both vulnerable to anthropogenic activities and slow to recover
	Biological productivity	Deep-sea sponges play important roles in the **biogeochemical cycling** and the habitat they create support diverse benthic ecosystems
	Biological diversity	Deep-sea sponge grounds provide a **habitat** to diverse benthic vertebrate and invertebrate species
	Naturalness	Anthropogenic activities such as oil and gas exploitation and mining are **impacting deep-sea sponge grounds**

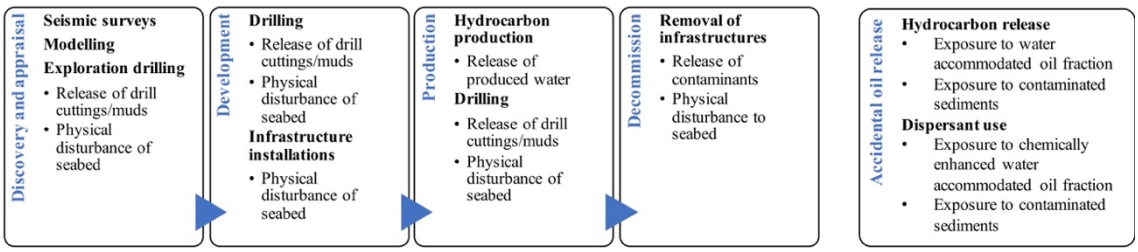

Figure 1-2. Flow chart of an oil field development process divided into four phases and main activities associated with each phase.

Physical disruption and increased sedimentation are also associated with the installation of pipelines, which export produced hydrocarbons onshore. Power transmission cable installations significantly impact local benthic communities inflicting a 100% mortality rates to glass sponges below the cables and a 15% mortality rate within 1.5 m of the cables all along its footpath (Dunham *et al.*, 2015) with potentially similar effects expected from pipeline deployments (OSPAR Commision, 2010).

Chapter 2 of this thesis will provide a study of the impact of oil and gas infrastructures (as well as fishery impacts) on deep-sea sponge grounds and the associated megafauna community in the Faroe-Shetland channel.

Discharges of drill cuttings and drill muds

In the early stages of drilling, drill cuttings and drill muds, comprising residual rock fragments from the well and drilling fluid chemicals, are released directly into the environment at depth (Ellis *et al.*, 2012). For the remainder of the drilling process, treated cuttings are typically discharged at the surface, from where they sink to the seafloor under the rig. Unless dispersed by active near-bed currents, drill cuttings can accumulate on the seabed and over time may release contaminants, especially if disturbed (OSPAR Commision, 2010). The usually customised drill muds can be classified into three types: oil-based, synthetic and water-based fluids all of which may contain toxic chemicals, including polyaromatic hydrocarbons (PAH) and metals. Only two studies have shown the impact of drilling mud and cuttings on megafaunal communities associated with sponge grounds, both in the North-East Atlantic (Gates and Jones, 2012; Jones *et al.*, 2012). Both studies indicate major reductions in sponge densities and reduced diversity close to drilling activities (100–200 m) that persist for several years after the drilling stops. At greater distance to the wells, impacts are reduced, and densities seem to increase, in most cases, with distance to the disturbance. Both studies also highlight the need for pre-drilling data to determine if the benthic communities are recovering (Figure 1-3).

The severity of the impact of drill muds and cuttings has been better studied on other benthic communities where the effects have been shown to depend largely on abiotic conditions such as depth and currents as well as the concentration of chemicals associated with the muds (Ellis *et al.*, 2012; Henry *et al.*, 2017). For synthetic and water-based muds, a decrease in community diversity and abundance has been measured up to 1000 m away from the release location (Ellis *et al.*, 2012). Functional changes in benthic communities associated with a loss of suspension-feeding species and an increase in

deposit feeders have also been detected at release sites (Trannum *et al.*, 2010; Ellis *et al.*, 2012). The spatial footprint of the impacts is largest during the first 1–2 years after drilling and then reduces in extent afterwards as drill muds have settled (OSPAR Commission, 2016). Today the production and release of oil-based drill muds have been widely reduced in the North-East Atlantic by the oil and gas industry, thanks to the development of processing technologies (OSPAR Recommendations R2001/1, 2006/5 and 2010/18, OSPAR Commision, 2010), but the use of oil-based drill muds in the past has been shown to have a local but strong and lasting impact on benthic communities (OSPAR Commision, 2010; Henry *et al.*, 2017). Potential impacts of past releases of oil-based drill muds on sponge grounds and associated benthic communities therefore still need to be understood.

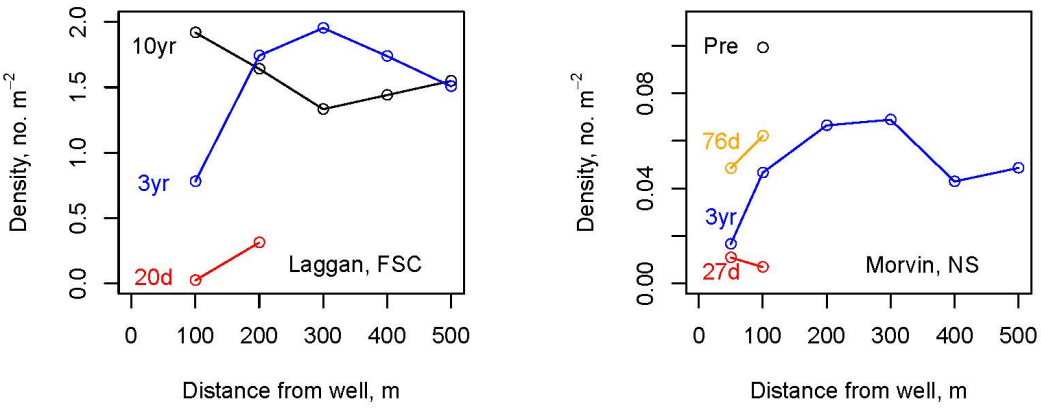

Figure 1-3. Field data on the initial impact and recovery from oil drilling disturbance in deep-sea sponges in the Faroe-Shetland channel (FSC), at the Laggan site (Jones *et al.* 2012), and Norwegian Sea (NS), at the Morvin site (Gates and Jones 2012). The density of all megafaunal sponges is shown with distance from drilling activity at different time points (colours) after drilling (years [yr] and days [d]). Pre indicates densities prior to drilling activity. Figure credits: DOB Jones (NOC Southampton).

Decommissioning

As offshore infrastructures age, decommissioning options for the physical removal of oil and gas infrastructure including pipelines, platforms, drill cuttings and the capping of wells need to be considered (Figure 1-2). Worldwide, there are over 7500 oil and gas structures offshore and about 85% of them will need to be decommissioned by 2025 (Fowler *et al.*, 2014). In the North-East Atlantic, the dumping, and leaving wholly or partly in place, of disused offshore installations has been prohibited within certain sea areas, under OSPAR Decision 98/3 on the Disposal of Disused Offshore Installations since 1998. Based on a predefined assessment demonstrating that there are significant reasons why an alternative disposal is preferable to reuse or recycling or final disposal on land, the competent authority of the relevant Contracting Party may authorise companies

to leave some parts of the installations in place after consultation with the other Contracting Parties. Such derogations concern very heavy concrete and steel installations which might provide a suitable settlement ground also for deep-water sponges. Until 2009, 122 offshore installations have been brought ashore for disposal and only 5 permits have been issued for structures to be left in place (OSPAR Commission, 2009a). However, with more and more installations approaching their end of life, the industry has started to lobby for a modification of the Decision itself instead of using the derogation options provided by OSPAR Decision 98/3. The argument is that the physical impact on the seabed as well as the economic costs of such operations are substantial.

Environmental impacts caused by a complete removal of offshore infrastructure that could negatively affect deep-sea sponge grounds and associated communities may include: contamination of the water column by hydrocarbons and other chemicals, direct damage to the seabed and smothering by increased sedimentation (Fowler *et al.*, 2014). Partial removal of offshore infrastructure could also impact the community associated with the infrastructures: damage to the sessile fauna close to the removal point is to be expected and natural vertical migrations of motile fauna along the oil rig could potentially be disturbed (Fujii and Jamieson, 2016). Decommissioning of oil and gas industry infrastructure has not yet taken place within known deep-sea sponge grounds and so potential impacts of decommissioning at community level is for the moment unknown. Under UK regulation, decommissioning impacts on the environment must be considered in the environmental impact assessment produced in the beginning of any new oil and gas field development (Department of Energy and Climate Change, 2011).

1.3.2. Accidental spills and releases

The Deepwater Horizon oil spill was one of the largest and deepest offshore oil spills to date, with approximately 3.19 million barrels of oil released into the water at a depth of 1500 m (Beyer *et al.*, 2016). It was also the first time dispersants were used to such an extent at depth to mitigate the formation of a surface oil slick that would have impacted upon sensitive coastal ecosystems (White *et al.*, 2014). Almost 3 million litres of dispersant CorexitTM 9500 was released near the well head (White *et al.*, 2014). A large amount of the oil released into the water column formed several subsurface oil plumes (Diercks *et al.*, 2010). The most significant subsurface plume extended for 35 km at approximately 1100 m depth (Camilli *et al.*, 2010). The Deepwater Horizon incident thus created a new kind of oil spill where deep-water ecosystems and habitats were

exposed to high concentrations of dispersed crude oil and dispersants (Peterson *et al.*, 2012).

Impact of accidental oil releases is better understood in shallow-water than in deep ecosystems. In shallow-water coastal environments, oil spills have shown both lethal (high mortality rate) and sublethal effects (carcinogenic and cytotoxic impacts) on benthic species leading to changes in community diversity, age structure and trophic interactions (Suchanek, 1993). Impact of oil spills on deep-sea benthic ecosystems is far less understood. After the Deepwater Horizon incident, significant decreases in macro- and meiofaunal diversity were detected up to 17 km away from the well (Montagna *et al.*, 2013). Other studies have shown high mortality rate of deep-water corals, colonial and pelagic tunicates, sea pens as well as glass sponges within a 2 km radius of the well, but no further result on deep-sea sponges is given (White *et al.*, 2012; Valentine and Benfield, 2013).

Long-term impacts of oil spills in shallow-water ecosystems often take the form of community structure anomalies (absence of organisms of a specific age class) owing to the longevity and slow growth rate of some species (Kingston, 2002). Long-term impacts of deep-sea oil spill such as the Deepwater Horizon oil spill remain unknown. Deep-sea sponges display relatively slow and strongly seasonal growth rates varying from a few millimetres to a couple of centimetres per year (Fallon *et al.*, 2010; Dayton *et al.*, 2013; Dunham *et al.*, 2015), suggesting that deep-sea oil spills in the vicinity of deep-sea sponge grounds could have a strong long-term community effect on these habitats.

1.4. Physiological and ecotoxicological effects on individual sponges

1.4.1. Main impacts of routine offshore oil and gas activities on deep-sea sponges

Seismic surveying during hydrocarbon exploration and appraisal phases

During the initial phases of exploration and appraisal, seismic surveys are conducted to assess subseafloor structures and determine drilling location (Department of Trade and Industry, 2001). Impact of seismic surveys on marine invertebrates and larval development and survival has been investigated in several studies (Aguilar De Soto *et al.*, 2013; Nedelec *et al.*, 2014). Developmental delays and malformations in scallops have been identified as potential effects of seismic surveys on benthic organisms (Aguilar De Soto *et al.*, 2013). In gastropods, seismic pulses decreased larval development and

increased mortality by over 20% (Nedelec *et al.*, 2014). However, no studies have yet investigated the effect of seismic surveys on sponges or their larval stages.

Sedimentation from seabed disturbance

The phases of offshore exploration and development are characterised by drilling and installation of heavy infrastructures, which are associated with resuspension of sediments that can affect local benthic organisms including deep-sea sponges (OSPAR Commission, 2010) (Figure 1-2). Bell *et al.* (2015) summarised the often species-specific effects of sedimentation on marine sponges, focusing mainly on shallow-water species. Increased sedimentation impacts sponge filtration and feeding (Reiswig, 1971; Bannister *et al.*, 2012), respiration (Lohrer *et al.*, 2006; Bannister *et al.*, 2012), reproduction (Roberts *et al.*, 2006) and growth (Roberts *et al.*, 2006; Wilkinson and Vacelet, 1979). Additionally, evidence of tissues sloughing in shallow-water sponge *Halichondria panicea* was found after exposure to increased sedimentation (Barthel and Wolfrath, 1989). Studies on deep-water sponges have confirmed some of the findings made on shallow-water sponges. Heavy sedimentation on deep-water sponge *Geodia barretti* led to a 50%–86% reduced respiration rate depending on sediment concentration tested but was associated with a fast recovery after exposure to sediments (Tjensvoll *et al.*, 2013; Kutti *et al.*, 2015). Furthermore, sedimentation caused a rapid arrest in feeding behaviour and chamber clogging in the two deep-sea glass sponges *Rhabdocalyptus dawsoni* and *Aphrocallistes vastus*. However, some aspects in the response of the two glass sponge species differed: feeding was resumed earlier in *A. vastus* and sediment level required to halt feeding was lower for *R. dawsoni* (Tompkins-Macdonald and Leys, 2008). This shows that increase in sedimentation has an overall negative impact on deep-sea sponges, with some species being more resilient than others.

Release of contaminants in the environment during routine operations

Routine operations during the production phase of an oil field development include the discharge to the sea of produced water that contains small amounts of hydrocarbons such as PAHs, dissolved metals and naturally occurring radioactive elements such as radium-226 and radium-228 (Figure 1-2) (Neff *et al.*, 2011). The overall volume of oil released into the North-East Atlantic through produced water discharges has been reduced following industry effort, through decisions such as the OSPAR recommendation 2001/1 (OSPAR Commission, 2010). However, produced water still remains the main source of hydrocarbons in the environment from oil and gas industry-linked activities (Neff *et al.*, 2011). Upon release, produced water is believed to be diluted

very rapidly into the ambient seawater (Neff *et al.*, 2011). Therefore, although some PAHs are persistent in the environment and can be toxic at higher concentration as discussed in the next section (for accidental releases of hydrocarbons), produced water is expected to have a very low impact on marine organisms (Neff *et al.*, 2011). However, PAHs from produced water could have sublethal effects on deep-sea sponges. Benthic suspension feeders such as mussels have been shown to accumulate PAHs when exposed to produced water (Sundt *et al.*, 2011). Moreover, low concentration of PAHs can be bioaccumulated in sponges at higher levels than mussels (Negri *et al.*, 2006; Batista *et al.*, 2013; Mahaut *et al.*, 2013; Gentric *et al.*, 2016). Changes in fatty acid content in sponges exposed to PAHs have also been observed. It has therefore been suggested to use sponges as environmental bioindicators for PAHs concentration monitoring (Batista *et al.*, 2013).

Dissolved metals can also be present in produced water including barium, iron, manganese, mercury and zinc. Shallow-water sponges are known to bioaccumulate zinc (Gentric *et al.*, 2016). It is consequently possible that deep-sea sponges could also bioaccumulate metals in their tissue from produced water exposition, but no study has been conducted so far on this subject. Notably, zinc naturally present in the environment has been shown to be incorporated into sponge spicules (Hendry and Andersen, 2013). However, no studies looking at the impact of metal concentration from anthropogenic sources in sponge spicules have been conducted so far.

Decommissioning

Removal of ageing offshore infrastructures during decommissioning could lead to an increase in sedimentation and a release of hydrocarbons and other chemicals into the marine environment (Figure 1-2) (Fowler *et al.*, 2014). Yet targeted disturbance experiments of the drill cuttings accumulated on the seafloor demonstrate no major effect on the spatial distribution of cuttings contamination or of the biological communities present at the seabed (OSPAR Commission, 2009b). It has to be born in mind, however, that the removal of large anchors or installations on the seafloor will likely cause resuspension of a much larger extent. Intensive water column and sediment monitoring will be required to assess the effects of the removal of individual or multiple installations.

As previously stated, no decommissioning project has yet taken place within deep-sea sponge grounds and so potential impacts of decommissioning at individual level are for the moment unknown. It can only be hypothesised that impacts on deep-sea

sponges associated with high sedimentation rate and hydrocarbon pollution described during the exploration, development and production phases could also occur during the decommissioning phase.

1.4.2. Impacts of accidental hydrocarbon release and dispersant use on deep-sea sponges

During accidental spills, large amounts of hydrocarbons are released directly into the marine environment. During oil spills, PAHs are of particular concern when considering ecotoxicological impacts on organisms present in the vicinity of the spill location (Blackburn *et al.*, 2014). In shallow-water sponges, high concentrations of PAHs have been shown to disturb sponge larval settlement and development (Cebrian and Uriz, 2007; Negri *et al.*, 2016). Effects of dispersants and dispersed oil on larval stages of various other marine organisms have been investigated but results of higher toxicity associated with the use of dispersant seem to depend on the organisms considered and the duration of exposition (Singer *et al.*, 1998; Epstein *et al.*, 2000; Stefansson *et al.*, 2016). In tropical corals, exposure to dispersed crude oil resulted in increased mortality in larvae of the coral *Stylophora pistillata* and a stronger decrease in larvae settlement rate compared to exposure to crude oil alone (Epstein *et al.*, 2000). Furthermore, exposure to dispersed oil and dispersants alone has led to a strong health decline (defined by percentage of live polyps and tissue coverage) in three deep-water coral species from the Gulf of Mexico (DeLeo *et al.*, 2016). No studies have yet tested the effects of dispersed oil or dispersants on marine sponges and sponge larvae.

Due to unexpected external circumstances, it was not possible to appropriately collect deep-sea sponges for experimental purposes during this thesis. Experimental work was therefore undertaken on shallow-water sponges to investigate some of the questions highlighted above. Chapters 3 will present experimental work conducted to determine the impact of crude oil and dispersed crude oil contaminated seawater on shallow-water sponge *H. panicea*.

Long-term impacts of a deep-sea oil spill could be derived from sediment-associated hydrocarbons. It is estimated that 35% of the oil released into the marine environment during the Braer oil spill off the Shetland Islands in the North-East Atlantic subsequently ended up in subtidal sediments (Davies *et al.*, 1997). PAHs and hydrocarbon breakdown are slowed down in sediments owing to overall anoxic conditions within the finely grained sediments (Atlas and Hazen, 2011). However, benthic organisms can be

exposed to sediment-associated PAHs or hydrocarbon via sediment resuspension. Bivalves are able to accumulate PAHs from the sediment during resuspension episodes (Nandini Menon and Menon, 1999). It has been suggested that deep-sea sponges can derive part of their nutrition from resuspended matter (Hogg *et al.*, 2010) and therefore could be impacted by PAH-contaminated sediments. Furthermore, Culbertson *et al.* (2008) showed that short-term and long-term exposure to 38-year-old residual petroleum associated with sediments led to a decrease in growth rate, lower health condition and decreased filtration rate in mussels. Dispersants have also been shown to persist in deep-sea sediments as dispersants were detected in sediments collected within deep-sea coral communities 6 months after the Deepwater Horizon spill (White *et al.*, 2014). This suggests that oil spills can have long-term impacts on deep-sea benthic organisms when hydrocarbons and dispersants enter the sediments, which is of concern for deep-sea sponges.

To investigate if sponges can be exposed to hydrocarbons through contaminated sediment resuspension, experimental work on shallow-water sponge *H. panicea* was conducted during this PhD. Chapter 4 of this thesis will present the results of the contaminated sediments experiments.

1.5. Effects on deep-sea sponges at cellular and molecular levels

1.5.1. Impacts of offshore oil and gas production activities on deep-sea sponges at cellular level

In the previous section, impacts of offshore hydrocarbon production activities on sponges at an individual level were summarised. These individual effects are due to biological disturbances at cellular and molecular levels which are reviewed in this section.

During the production phase of an oil field development, the release of drill muds has been found to impact deep-sea sponges at a cellular level (Edge *et al.*, 2016). Baryte, one of the major solid components of these drill muds, has been shown, in the deep-sea sponge *G. barretti*, to decrease lysosomal membrane stability (widely-used biomarker for cellular damage) (Edge *et al.*, 2016).

Hydrocarbon contamination including PAH pollution is also a main concern when considering cellular impacts of offshore oil and gas activities on sponges. Water-accommodated oil fraction (solution of soluble hydrocarbons in seawater) can activate the mitogen-activated protein kinase (MAPK) and apoptosis pathways in the sponge

Suberites domuncula (Châtel *et al.*, 2011). The MAPK pathway plays an important role in the cellular response to environmental and oxidative stress (Regoli and Giuliani, 2014). Increased DNA damage was also detected in *S. domuncula* (Châtel *et al.*, 2011). This study by Châtel *et al.* (2011) thus confirmed previous work conducted by Zahn *et al.* (1981, 1983), which also detected PAH-induced DNA damage in the shallow-water sponge *Tethya lyncurium*.

The cytochrome P450-dependent monooxygenase system has also been shown to be involved in the detoxification of PAH benzo[a]pyrene, in two marine sponge species (Solé and Livingstone, 2005). Lower yields of cytochrome P450 protein were detected in sponges compared with other Phyla (Cnidaria, Mollusca, Annelida, Arthropoda, Echinodermata and Chordata), but this could result from overall lower metabolic rates (Solé and Livingstone, 2005). Under PAH-contaminated conditions produced in the laboratory, PAH molecules interact with the aryl hydrocarbon receptor and induce the cytochrome P450 pathway (Regoli and Giuliani, 2014). The cytochrome P450 pathway is known to play an important role in oxidative stress responses (Solé and Livingstone, 2005), which are induced in many organisms after exposure to PAHs (Nebert *et al.*, 2000; Puga *et al.*, 2002; Regoli and Giuliani, 2014). Oxidative stress is a consequence of an imbalance in the antioxidant system in an organism. Normal aerobic metabolism produces reactive oxygen species (ROS), which are neutralised by the antioxidant system. Exposure to xenobiotic compounds can increase the formation of ROS and decrease the antioxidant system's functioning. Formation of ROS, in turn, downregulates the cytochrome P450, which limits the organism's capacity to deal with contaminants such as PAHs (Regoli and Giuliani, 2014). The role of the aryl hydrocarbon receptor in organisms impacted by oil spills was recently confirmed in a transcriptomic study showing an induction of stress response genes such as the aryl hydrocarbon receptor and the glutathione-S-transferase in oysters deployed during the Deepwater Horizon oil spill (Jenny *et al.*, 2016). However, no studies have reported the activation of the aryl hydrocarbon receptor and cytochrome P450 pathway in deep-sea sponges.

Dispersants themselves have been shown to trigger cellular stress responses in different organisms. In the commonly used model organism *Caenorhabditis elegans* (Nematoda), exposure to dispersant CorexitTM 9500A caused the abnormal expression of 12 genes, involved in a wide range of biological processes ranging from egg laying to neurological functions and oxidative stress (Zhang *et al.*, 2013). However, in the tropical coral *Montastraea franksi,* CorexitTM 9527 exposure led to increased expression of

genes coding for P-glycoprotein, heat shock protein 70 and heat shock protein 90 and, to a lesser extent, proteins involved in other cellular stress responses (Venn *et al.*, 2009). Furthermore, exposure to dispersants alone as well as dispersed crude oil led to an increase in cell membrane damages in diatoms, which was not observable in diatoms exposed to oil alone (Hook and Osborn, 2012). No studies so far have investigated the impact of dispersants on marine sponges.

As previously mentioned, the impact of crude oil and dispersed crude oil contaminated seawater or sediments at molecular level was investigated in shallow-water sponge *H. panicea* during this project and the results are available in chapters 3 and 4.

1.5.2. Impacts of offshore oil and gas production activities on deep-sea sponge-associated microorganisms

Sponges host highly diverse microbial communities often compared for its complexity to the bacterial community of the mammalian gut (Hentschel *et al.*, 2012). Although bacteria generally dominate deep-sea sponge microbial communities, eukaryotic and archaeal symbionts have also been described. Mainly found in the mesohyl of sponges, these microbes are metabolically very active and are believed to play important roles in the nitrogen and carbon metabolism (Li *et al.*, 2014). Deep-sea sponges are a rich source of secondary metabolites of great interest as new therapeutic compounds, and it is often the associated microbial communities that synthesise these compounds. Sponges' secondary metabolites show properties that include antifouling, antifungal, antibacterial or antiviral characteristics and are believed to play a major role in sponge defence against diseases or against other benthic organisms competing for the same substrata (Sipkema *et al.*, 2005).

The impact of environmental pollution and specifically exposure to hydrocarbons on the sponge-associated microbial communities are currently unknown. Studies have investigated the stability of shallow-water sponge associated microbial communities when exposed to thermal stress, changes in seawater pH or to high metal concentrations (Webster and Hill, 2001; Webster *et al.*, 2008; Selvin *et al.*, 2009; Fan *et al.*, 2013; Fang *et al.*, 2013; Tian *et al.*, 2014). However, only a few of these studies found, under stressed conditions, a shift in the associated microbial community composition (Webster and Hill, 2001; Webster *et al.*, 2008; Fan *et al.*, 2013; Tian *et al.*, 2014). A change in associated microbes was also correlated with a decline in overall sponge host health status characterised by an increase in sponge tissue necrosis and increased expression of genes

linked to cellular oxidative stress (Webster and Hill, 2001; Webster *et al.*, 2008; Fan *et al.*, 2013; Tian *et al.*, 2014). An oil-degrading surfactant biosynthesis gene has been isolated from bacteria associated with the shallow-water sponge *Acanthella sp.* (Anburajan *et al.*, 2015). However, the capacity of the bacteria to synthesise the surfactant when associated with the marine sponge or when exposed to crude oil was not investigated (Anburajan *et al.*, 2015). In the Gulf of Mexico, the deep-sea sponge *Myxilla methanophila* growing on tubeworms near cold seeps was found to be associated with putative oil-degrading bacteria after sequencing of its associated microbial community (Arellano *et al.*, 2013). In this case, it was hypothesised that the sponge had acquired the symbiont from its environment naturally rich in hydrocarbons (Arellano *et al.*, 2013). Whether the oil-degrading bacteria played a role in hydrocarbon detoxification or in sponge nutrition was not investigated (Arellano *et al.*, 2013). The capacity of deep-sea marine sponges to acquire oil-degrading bacteria after an oil spill event has not yet been investigated.

Changes in *H. panicea* associated bacterial community after exposure to crude oil contaminated sediment has been investigated and results are available in chapter 4.

1.6. Conclusion

Oil and gas activities are, today, taking place in deeper settings and impact deep-sea ecosystems. Oil and gas production activities impact deep-sea sponges and the habitats they form at all stages of an oil field development and at community, individual and cellular levels as summarised in table 1-2. At community level, physical disturbance and discharge of drill muds have been shown to decrease diversity and density of organisms associated with deep-sea sponge grounds. At individual level, physical disturbance and increased sedimentation inhibit the filtration systems of deep-sea sponges, while the discharge of produced water and drill cuttings could lead to bioaccumulation of hydrocarbons and metals (as shown in shallow-water sponges). At cellular and molecular levels, discharge of drill muds and produced water could trigger cellular stress responses, as shown in shallow-water sponges exposed to PAH and metal-contaminated seawater. Accidental releases of hydrocarbons and the use of dispersants during oil spill could result in benthic diversity decrease, individual sponge mortality and larval settlement disruption as well as trigger oxidative stress. However, most of the possible impacts described in this chapter have not yet been studied in deep-sea sponges.

The aim of the work conducted during my PhD project is to address some of the knowledge gaps highlighted above and in summary table 1-2. Chapter 2 will detail a spatial analysis of industry-led environmental surveys to determine the impact of oil and gas infrastructures as well as fishing activities on deep-sea sponge grounds in the Faroe-Shetland channel. Chapter 3 and 4 will present experiments conducted on model shallow-water sponge *H. panicea* exposed to contaminated seawater and sediments. Finally, chapter 5 will provide a summary and conclusion to the work undertaken within this PhD project, present an updated version of table 1-2 as well as discuss opportunities for future research.

Table 1-2. Overview of major impacts of offshore oil and gas activities on deep-sea sponges and deep-sea sponge grounds at community, individual, cellular and molecular levels, throughout an oil field development. Impacts described in deep-sea sponge species are highlighted in green. Impacts described in shallow-water sponge species but not yet confirmed for deeper species are highlighted in orange. Impacts described in other benthic organisms but not yet investigated in any sponge species are highlighted in red to emphasize current knowledge gaps.

		Exploration and appraisal	Field Development	Production	Decommissioning	Deep-sea oil spill
Community level	*Main concern*	*Physical disturbance of seabed and increase sedimentation*		*Discharge of drill muds and cuttings*	*Removal of structure*	*Exposure to high hydrocarbons and dispersant concentrations*
	Impacts				Benthic habitat destruction.	Changes in benthic community abundance, diversity, age structure and trophic interactions.
		Diminished benthic community.		Benthic community diversity/abundance decrease.		
Individual Level	*Main concern*	*Seismic survey and increase sedimentation*	*Increase sedimentation*	*Discharge of produced water*	*Release of chemical contaminants*	*Exposure to high hydrocarbons and dispersant concentrations*
	Impacts	Larval development delay and malformations.				Health decline, hydrocarbon bioaccumulation.
		Changed respiration rate and reproduction capacities. Decreased growth rate.		Bioaccumulation of PAH and heavy metals.		Larval settlement disturbance. Hydrocarbon bioaccumulation.
		Paused filtration.				
Cellular & Molecular levels	*Main concern*	*Discharge of drill muds and exposure to chemicals via release of produced water*				*Exposure to high hydrocarbons and dispersant concentrations*
	Impacts	Decrease immune system function.				Decreased immune system function.
		Activation of MAPKs and cytochrome P450 pathways. Oxidative stress.				Activation of MAPKs and cytochrome P450 pathways. Oxidative stress.
		Decrease of lysosomal membrane stability.				

Chapter 2 Influence of Anthropogenic and Environmental Factors on Deep-Sea Megafauna and Deep-Sea Sponges in the Faroe-Shetland Channel

2.1. Overview

This chapter aims at understanding the spatial scale and depth of impacts of both hydrocarbon production activities and fishing activities on deep-sea sponge grounds in the Faroe-Shetland channel. This chapter was recently submitted for publication in Scientific Reports (see appendix A).

2.2. Introduction

Ecological communities and processes such as recruitment or dispersal vary over temporal and spatial scales (Wiens, 1989). Understanding appropriate scales for observing ecological processes, as well as determining the origin of spatial variation (biological, environmental or anthropogenic), is very important to determine how ecosystems and communities function (Wiens, 1989; Borcard and Legendre, 2002; Borcard *et al.*, 2018). Changing sampling scale when analysing the same ecological assemblage can lead to drastically different findings (Nogués-Bravo *et al.*, 2008) and spatial analysis has been increasingly used in ecology in recent decades (Dale and Fortin, 2014). This is particularly important with recent fast temporal changes observed in ecosystems as a consequence of human activities and global climatic change (Halpern *et al.*, 2008).

Determining spatial structure in complex ecological community datasets is challenging but a range of methods have been developed. Distance-based Moran's eigenvectors mapping or dbMEM is one method that can be applied to determine spatial structures in the composition of ecological communities (Borcard and Legendre, 2002). It creates new variables or eigenfunctions that correspond to the spatial scales perceivable in the community composition (Borcard *et al.*, 2004). Initially called principal coordinates of neighbour matrices (PCNM), dbMEM provides eigenfunctions calculated solely on geographical coordinates and pairwise distances between sampling points (Borcard and Legendre, 2002). After extraction of positive eigenfunctions maximising Moran's index of spatial autocorrelation (Moran's I), dbMEM can help distinguish between spatial changes owing to community processes and spatial changes resulting from variations in environmental factors (Dray *et al.*, 2006). dbMEM analysis is a powerful tool for ecologists trying to understand the importance of spatial scales and can be combined with ordination analysis (Dray *et al.*, 2006) such as principal component analysis (PCA) and redundancy analysis (RDA). For example, when applied to cold-water coral reefs, dbMEM and PCA analysis showed that broad-scale variation in species assembly was related to environmental conditions such as bathymetry and hydrology

while small-scale variation in species assembly was explained by ecological characteristics such as species recruitment strategies (Henry *et al.*, 2013).

Environmental factors such as substrate type, depth, hydrography and food availability influence the distribution of deep-sea sponges (Klitgaard and Tendal, 2004; Kahn *et al.*, 2012; Knudby *et al.*, 2013; Beazley *et al.*, 2015; Murillo *et al.*, 2016). Anthropogenic factors, however, can also alter deep-sea sponge distribution (Bett, 2001; Jones *et al.*, 2006, 2007ab 2012; Clark *et al.*, 2016). As described in the first chapter of this thesis, impacts of hydrocarbon activities, from the drilling process to decommissioning activities, on deep-sea sponges and deep-sea sponge grounds include: high mortality rates closest to the drilling location, ceasing of filtration activities and changes to cellular stability, mostly resulting from increased sedimentation and release of drill cuttings and drill muds near the wells. However, deep-sea sponges are also highly vulnerable to fishing activities, including trawling and bottom longlines (Durán Muñoz *et al.*, 2012; Clark *et al.*, 2016; Murillo *et al.*, 2016).

In the FSC sponge grounds are found occurring at depths of around 500 metres. They are of the boreal ostur type formed by accumulations of demosponges, particularly *Geodia spp* (Bett, 2001; Klitgaard and Tendal, 2004). In July 2014 a United Kingdom nature conservation marine protected area (NCMPA) was established in the FSC to protect deep-sea sponge grounds. Nevertheless, fishing activities and trawling have been recorded within the FSC prior to the NCMPA designation (Bullough *et al.*, 1998; Bett, 2001). Oil exploration and production activities have also taken place in the FSC, within and around the NCMPA, since the early 1990s with the initial discovery of the Foinaven, Schiehallion and Loyal fields (Leghorn *et al.*, 1996; Leach *et al.*, 1999; Austin *et al.*, 2014) and the continued development of the Quad 204 project (Rees and Parke, 2013).

Most studies on deep-sea ecosystems consider the impacts from individual human activities at individual locations. However, studies considering multiple anthropogenic activities over larger spatial scales are needed to better understand their impacts on deep-sea sponges and benthic communities. Here, seabed still images and environmental data from a range of academic and industrial sources have been used to conduct a community-level spatial analysis of sponges and associated megafauna in the FSC. The objectives of this chapter were to: (1) successfully combine multiple industry-led environmental surveys to complete a community level analysis, (2) untangle the impacts of environmental and anthropogenic factors on the distribution of megafauna including

sponges in the FSC and (3) determine at which spatial scales these environmental and anthropogenic factors influence local megafauna communities.

2.3. Material and methods

2.3.1. Sampling area

Still images from environmental monitoring surveys conducted at six sites within the FSC between 2002 and 2014 were accessed for this study: Clair, Foinaven-Schiehallion-Loyal, Laggan, Rosebank (along a proposed pipeline route), Suilven and William (Figure 2-1AB, Table 2-1). The FSC is located between the Scottish and Faroese continental shelves. To the southwest, the FSC reaches depths of about 850 m and is separated from the North Atlantic Ocean by the Wyville-Thomson ridge. To the northeast, the FSC is open to the Norwegian Sea and deepens to almost 2000 m (Bullough *et al.*, 1998).

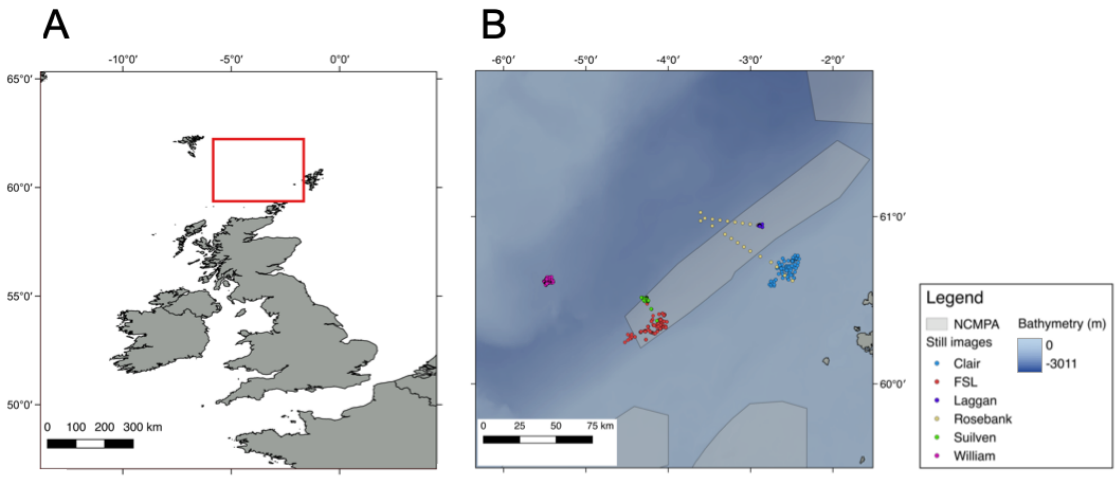

Figure 2-1. Location of the study area and of the still images analysed. (A) Location of the FSC highlighted in red. (B) FSC NCMPA with the location of the still images used in this analysis coloured by field (see legend).

In total, 4665 still images from transects conducted with remotely operated vehicles were catalogued and 2436 still images were selected for further analysis (Figure 2-1, table 2-1). As images originated from 16 different surveys (Table 2-1), image selection was strict to only include images of the best quality. Images partly obstructed by sediments, too far away from- or too close to the seabed (below 1 m and beyond 2 m), and images of poor resolution (less than 300 dpi) were not included in the analysis. Furthermore, since each survey covered different spatial areas, site will not be considered in this study as a variable but rather be used as parameter determining the origin of each still image.

2.3.2. Image analysis

For each still image, all clearly visible megafauna were recorded, and organisms were identified. Species records were then combined into operational taxonomical units (OTUs) to limit the number of rare taxonomical groups, which could bias the statistical analysis of the data (Legendre and Gallagher, 2001). Presence/absence data was gathered in this study to minimise bias as the still images originated from different surveys (with different cameras, remotely operated vehicles and scaling methods). In total, 17 non-sponge OTUs were considered in the analysis: Actinaria (Act), Alcyonacea (Alcyo), Asteroidea (Ast), Bivalvia (Biv), Bryozoa (Bryoz), Cirripedia (Cirr), Crinoidea (Crin), Hydrozoa (Hydroz), Pennatulaea (Pen), Crustacea (Crust), Echinoidea (Echin), Gastropoda (Gastrop), Holothuria (Holoth), *Molva spp,* Ophiuroidea (Oph), Other Fishes (Oth fish) and Polychaeta (Polych) (Figure 2-2). As sponge identification requires biological samples and examination of their spicules, sponge OTUs were based on morphology alone. Hence erect sponge OTUs were grouped into the following six morphological groups defined in the Thesaurus of Sponge Morphology (Boury-Esnault and Rützler, 1997): arborescent (Arb) e.i. branching, clathrate (Clath) e.i. lattice-forming, flabellate (Flab) e.i. fan-shaped, globular (Glob), massive (Mass) and other erect sponge morphotypes (Oth spo) (Figure 2-2). Two other OTUs were constituted for the encrusting sponges (Enc) and cushion-shaped sponges (Cush) respectively (Figure 2-2).

Dominant substrate type was also determined for each still image. Substrate types were categorised into the following five groups: (1) sand, (2) cobble/gravel, (3) sand with boulder, (4) cobble/gravel with boulder and (5) boulder, adapted from the Wentworth scale (1922). Finally, presence of object and trawl marks as identified by Roberts *et al.* (2000) as distinctive linear scars on the seabed was recorded on each image (Figure 2-3).

Table 2-1. Summary of still images processed.

Field	Location	Depth (m)	Numbers of surveys included in analysis	Year of survey(s)	Number of images available	Number of images retained in the analysis
Clair	60°68N, 2°51W	123-176	4	2000, 2004, 2010, 2013	920	470
Foinaven-Schiehallion-Loyal	60°34N, 4°18W	361-925	8	2002, 2003, 2005, 2006, 2007, 2011, 2015, 2016	1525	935
Laggan	60°94N, 2°91W	567-601	1	2007	1322	490
Rosebank	60°99N, 3°54W	129-1118	1	2011	115	78
Suilven	60°51N, 4°27W	455-879	1	2002	181	66
William	60°62N, 4°27W	749-800	1	2006	601	397

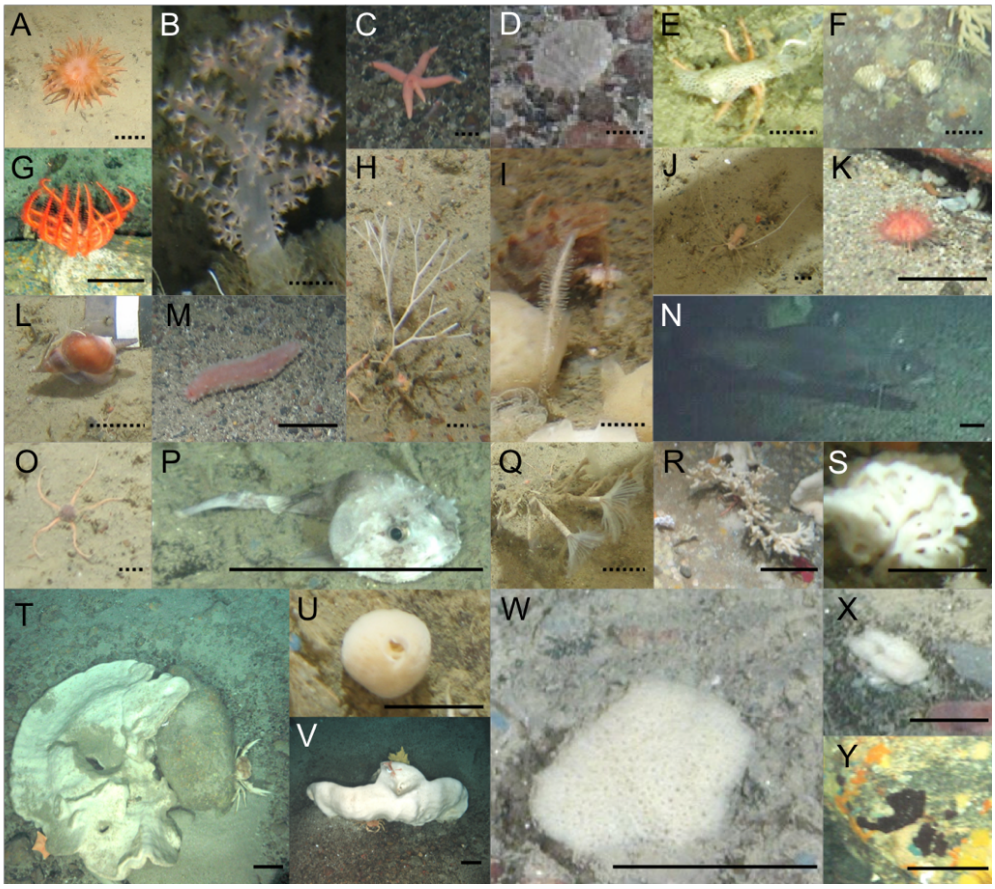

Figure 2-2. Operational taxonomical units (OTUs) considered in this study. (A) Actinaria (Act). (B) Alcyonacea (Alcyo). (C) Asteroidea (Ast). (D) Bivalvia (Biv) (E) Bryozoa (Bryoz). (F) Cirripedia (Cirr). (G) Crinoidea (Crin). (H) Hydrozoa (Hydroz). (I) Pennatulaea (Pen). (J) Crustacea (Crust). (K) Echinoidea (Echin). (L) Gastropoda (Gastrop). (M) Holothuria (Holoth). (N) *Molva spp.* (O) Ophiuroidea (Oph). (P) Other Fishes (Oth fish). (Q) Polychaeta (Polych). (R) Arborescent sponge (arb). (S) Clathrate sponge (Clath). (T) Flabellate sponge (Flab). (U) Globular sponge (Glob). (V) Massive sponge (Mass). (W) Other erect sponges (Oth Spo). (X) Cushion sponges (Cush). (Y) Encrusting sponges (Enc). Scale bars: full scale bare 10cm, dashed scale bar 2cm.

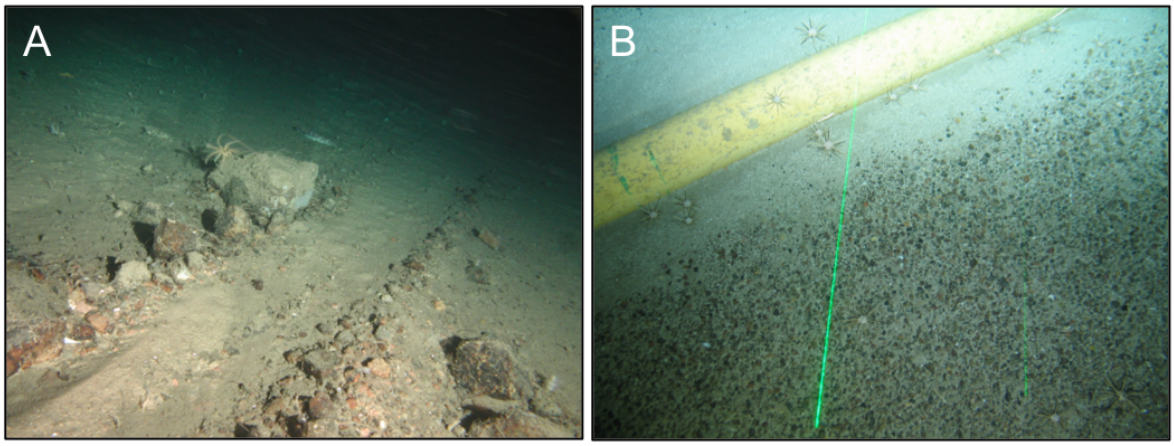

Figure 2-3. Evidence of anthropogenic activities taking place in the Faroe-Shetland channel (FSC). (A) Trawl marks visible in the seabed. (B) Pipeline as an example of an object detected in this study (lasers separated by 35cm).

2.3.3. Environmental and anthropogenic factors

In order to assess the importance of environmental and anthropogenic variables on the distribution of benthic megafauna in the FSC, data from several online resources were accessed. Slope, aspect and rugosity were extracted from bathymetry shapefile available at the general bathymetry charts of the Oceans (GEBCO; https://www.gebco.net, spatial resolution 30 arc second), using the freely available software QGIS (QGIS development Team, 2018). Aspect was subsequently converted into northness and eastness, as follows:

$$(1)\ Eastness = \sin(Aspect)$$

$$(2)\ Northness = \cos(Aspect)$$

Average annual temperature and salinity, variance of annual temperature and salinity as well as neap and spring seabed stress (parameters determining seabed disturbance arising from tidal currents) were made available through the Atlantic Interactive project (http://gtr.ukri.org/projects?ref=NE%2FM007235%2F1; unpublished data). Spring and neap estimates of bed stress were estimated from the NEMO simulation AMM60 with a 1.8 km resolution for the domain 40.1N to 64.9N, and 24.9W to 17.3E (Guihou and Polton, 2015; Guihou *et al.,* 2018). Surface and bed temperature and salinity data from 2000 - 2009 were modelled from a POLCOMS Atlantic Margin Model with ~12 km resolution and domain 40° 6'N 19° 49'W - 64° 53'N 13° 0'E (footnote available at https://www.bodc.ac.uk/data/documents/nodb/316641/; Holt *et al.,* 2012).

Demersal fishing effort per ICES (International Council for the exploration of the Sea) rectangle (spatial resolution 0.5° latitude and 1° longitude) in the form of annual landings in tonnes was acquired through the Marine Scotland open data network initiative national marine plan interactive (NMPI). Demersal fishing effort was available from 2012 to 2016 but an average over the four years was used in this study. Some of the still images were located outside of the demersal fishing effort shapefile spatial extent but the data was included regardless.

In order to determine the impact of oil and gas infrastructure and drilling process on the benthic megafauna communities in the FSC, distance to the closest well and distance to the closest pipeline were calculated for each still image with the R package

Geosphere (Hijmans, 2017) from Oil and Gas UK shapefiles available for visualisation on the NMPI website.

A summary of the variables considered in this study is given in table 2-2. Although most environmental and anthropogenic factors used in this study are at low resolution, these data are of the best quality currently available. The use of images over a large spatial area (not just focussed over a single field but over several) enabled me to extract ranges of values for each environmental and anthropogenic factor and to consider them in the analysis. Furthermore, in order to eliminate any statistical bias in the analysis, correlations between environmental variables were determined, using the R package corrplot (Wei and Simko, 2017). Slope, salinity, variance of salinity and variance of temperature were removed from the analysis as these variables showed strong correlation with ruggedness and temperature respectively (Figure 2-4). Furthermore, a stepwise selection step on consecutive RDA models further determined the significant environmental and anthropogenic variables to be used in the statistical analysis. In total, eight environmental variables and five anthropogenic variables were used in the statistical analysis step (Table 2-2).

Table 2-2. Environmental, anthropogenic and spatial variables considered in the redundancy analysis (RDA) models. Variables selected for the final PCA and pRDA models are highlighted in bold.

Environmental variables	Anthropogenic variables	Spatial variables
Depth	**Presence of objects**	**MEM1 and MEM15**
Substrate type	Presence of trawl marks	MEM2 to MEM14
Average annual temperature	**Distance to closest well**	
Neap tide seabed stress	Distance to closest pipeline	
Spring tide seabed stress	**Demersal fishing effort**	
Eastness and Northness		
Ruggedness		

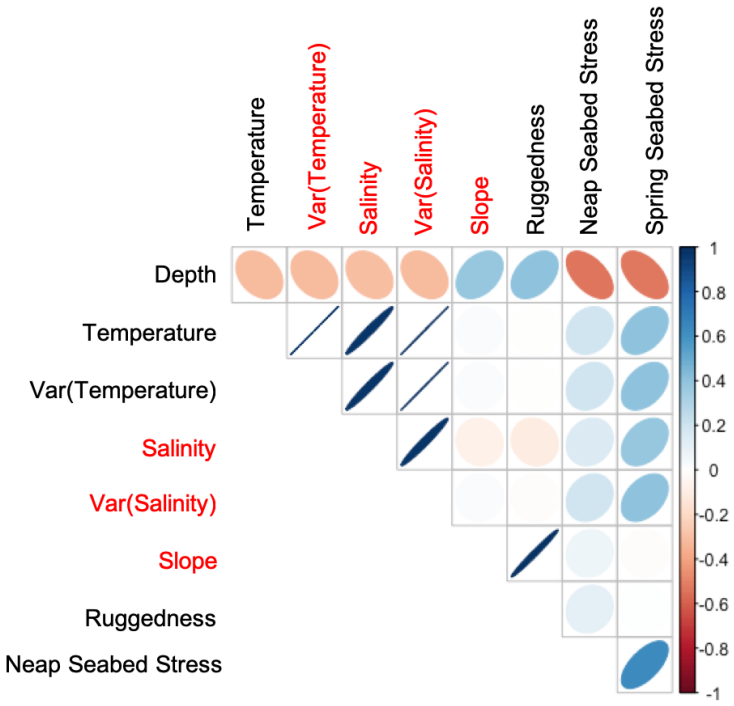

Figure 2-4. Correlation plot of available continuous environmental variables. Var() is for variance of. Variables in red have been removed from the analysis as they show strong correlations with other factors.

2.3.4. Statistical analysis of the data

OTUs presence data at each still image location were organised into an OTU presence/absence matrix in which each line of the matrix corresponds to a single still image location and each column record the presence or absence of a specific OTU. Hence single photographs were chosen as the sampling unit here. Individual sampling units typically had abundances of tens of individuals, which was considered to be sufficient for robust analysis. Furthermore, dbMEM is robust to zero-inflated community data. Environmental and anthropogenic data were similarly organised into an environmental matrix. Finally, a spatial matrix was also constructed with fifteen positive spatial eigenvectors extracted through dbMEM conducted with the R package adespatial (Dray *et al.*, 2018). Owing to the resolution of our dataset, the first eigenvectors created through this analysis will describe large spatial scale changes, (in this study over about 50 km (so across fields)) while the last eigenvectors will relate to small spatial scale changes (here about 1km (within field changes)). A forward step-wise selection was then applied to retain the most relevant eigenvectors, which were organised into a spatial matrix.

Selection of statistically significant spatial, environmental and anthropogenic variables was then conducted through the construction of a RDA model. RDA is an

extension of principal component analysis (PCA) in which the response variables (here megafaunal composition) can be modelled as a function of multiple explanatory variables (Zuur *et al.*, 2007). For this step, a subset of the data (1361 still images) was used in order to remove missing values (as some environmental data were not available for the whole set of images). Hellinger standardisation was applied on the OTU matrix to reduce the weight of rare OTUs (present in fewer images) and to maintain linear relationships between OTUs and environmental/anthropogenic variables (Legendre and Gallagher, 2001). In the Hellinger standardisation, each measurement (here presence/absence of a specific OTU) is divided by the corresponding row sum (the site of the measurements, here the specific still image) before a square root transformation is applied (Borcard *et al.*, 2018).

To investigate relationships between selected explanatory variables, a PCA was undertaken and visualized using the R package factoextra (Kassambara and Mundt, 2017). Finally, to investigate the influence of specific environmental and anthropogenic factors on the megafauna composition, a partial RDA (pRDA), in which spatial parameters were used as conditions and environmental and anthropogenic variables were included as constraints, was performed. This pRDA was built on the whole dataset. The RDA and pRDA were conducted with R package vegan (Oksanen *et al.*, 2017). All the statistical analysis steps were conducted in RStudio (R Core Team, 2017). A statistical workflow is provided in figure 2-5 to summarise the statistical analysis steps undertaken in our study.

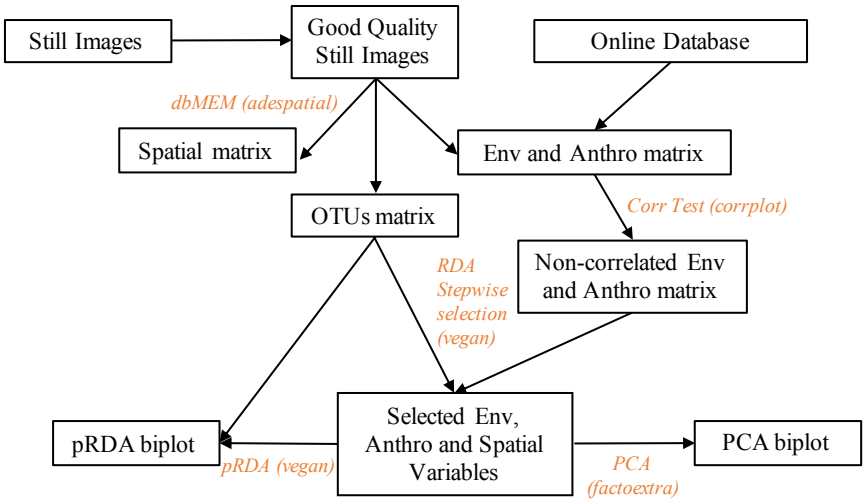

Figure 2-5. Statistical workflow followed in the study. The statistical steps executed and R package (in brackets) used are highlighted in orange. Env stands for environmental and anthro stands for anthropogenic.

2.4. Results

2.4.1. Gross observations

Differences between OTU prevalence within the FSC were detected in this study. Overall encrusting sponges, Crustacea and Echinoidea were the most frequently recorded OTUs and were detected on 26.7%, 19.3% and 16.3% of the still images respectively (Figure 2-6A). Flabellate sponges, globular sponges and massive sponges as well as Ophiruiroidea were also regularly encountered (recorded on 14.7%, 13.3%, 13.3% and 15.3% of the stills analysed respectively). Clathrate sponges and Holothuria were the two rarest OTUs considered in this analysis as they were only detected on 2.1% and 2.8% of the images respectively (Figure 2-6A). Differences in OTUs prevalence between sites could also be observed. Notably, arborescent sponges were almost exclusively encountered at Foinaven-Schiehallion-Loyal (12.6%). Flabellate and massive sponges as well as Bivalvia, Cirripedia, Crustacea, Echinoidea and *Molva spp* were also recorded in proportionally more still images from Foinven-Schiehallion-Loyal than from the other sites (Figure 2-6A).

Dominant substrate type also differed between FSC areas. Gravel/cobble and sand were the most frequent substrate types recorded in this study (Figure 2-6B). Sand was dominant at Clair, Rosebank and William whereas gravel/cobble was the most frequent substrate type at Foinven-Schiehallion-Loyal and Laggan. Hard substrate formed by boulders was predominantly recorded at Suilven (Figure 2-6B). 92% of the images analysed did not contain any objects. Pipelines were recorded on 3.1% of the still images while chains and ropes were present on 2.5% of the still images. No objects were detected at Rosebank, Suilven and William. On the contrary, Foinaven-Schiehallion-Loyal appeared to be the most developed site with 18.8% of images including an object (Figure 2-6C).

Trawl marks were rare in our study. Overall 1.7% of the images displayed trawl marks (Figure 2-6D). This proportion reached a maximum of 17.4% at William. No trawl marks were recorded at Rosebank and Suilven (Figure 2-6D).

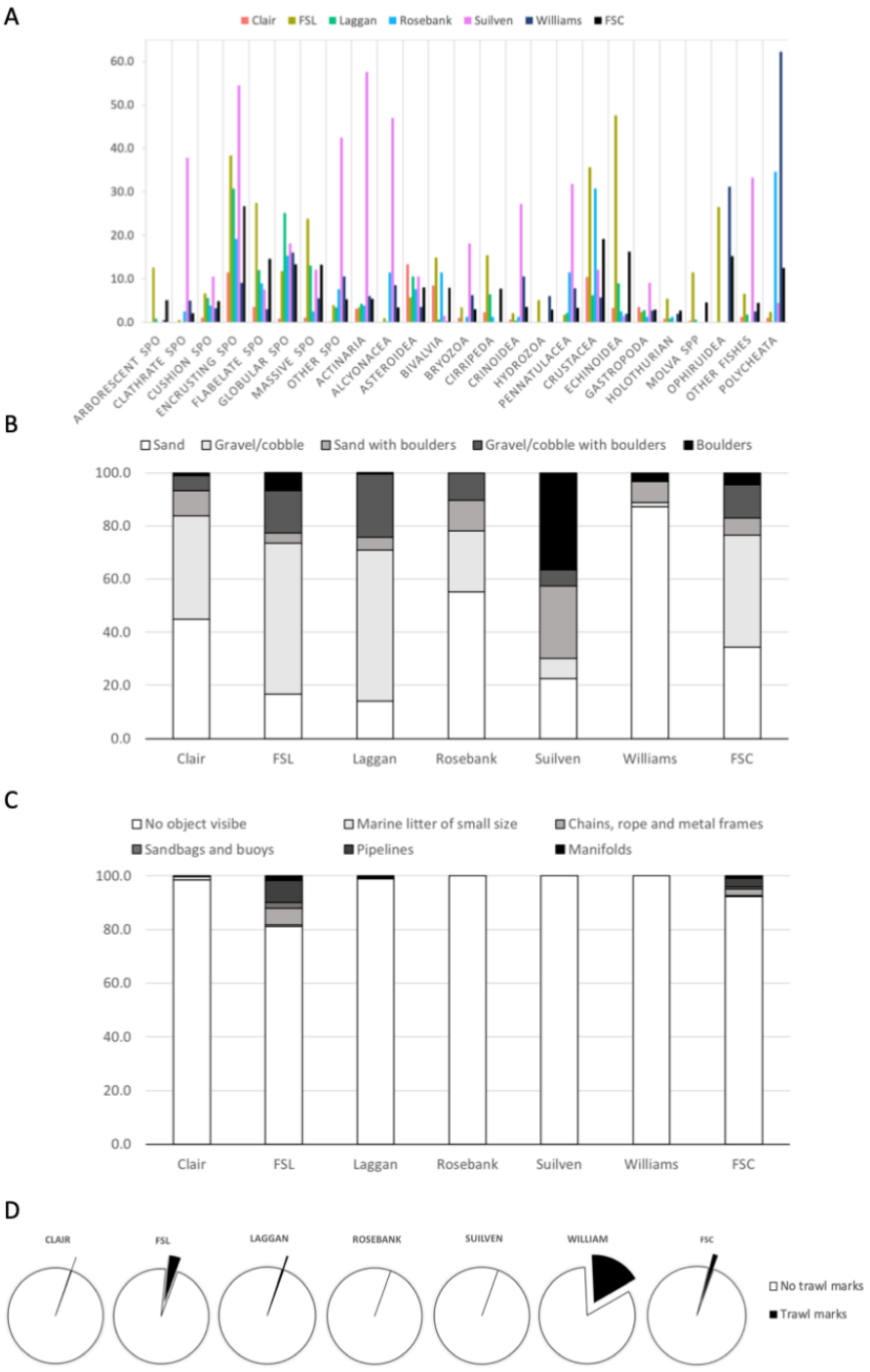

Figure 2-6. Overall results of the image analysis (A) Prevalence of each OTUs at all sites (% still images in which each OTU was recorded). (B) Proportion of dominant substrate types recorded at each site. (C) Proportion of still images with object recorded at each site. (D) Proportion of still images with and without trawl marks.

2.4.2. Selection of spatial, environmental and anthropogenic variables

Following the dbMEM, a forward stepwise selection procedure determined that only 3 eigenvectors significantly contributed to explain megafauna composition variation in the FSC. The two largest-scale eigenvectors MEM1 and MEM2 representing spatial scales at several tens of kilometers (larger distance between images) as well as the finest-scale MEM15 representing spatial scales of hundreds of meters were therefore retained for further variable selection (Table 2-2).

A RDA model using all available environmental and anthropogenic variables as well as the three pre-selected MEM eigenvectors was then performed, followed by another stepwise selection procedure (Table 2-2). Eight explanatory variables were retained for further analysis due to their significant effect on megafaunal distribution and included: substrate type, substrate ruggedness, temperature, presence of objects, distance to well and fishing effort as well as MEM1 and MEM15 (Tables 2-2 and 2-3).

2.4.3. Spatial scales of environmental and anthropogenic variables

A PCA was performed with the explanatory matrix determined above to investigate the relationship between the spatial eigenvectors MEM1 (broad scale) and MEM15 (fine scale) with the environmental and anthropogenic variables selected (Figure 2-7). On the biplot, MEM1 and MEM15 appear orthogonal as expected since these eigenvectors respectively represent the broadest and the finest scale spatial autocorrelation identified in this study. Data points are well scattered around on the biplot in both MEM1 (broad scale) and MEM15 (fine scale) directions, highlighting that megafauna composition changes at both broad and fine scales. MEM1 (broad scale) runs almost parallel to the first axis of the PCA biplot and, thus has the largest overall effect. Temperature, distance to well and fishing effort are more related to MEM1 (broad scale) than any other spatial eigenvectors whereas substrate type and ruggedness are more related to MEM15 (fine scale). The object type vector is oriented between the two MEM eigenvectors which suggested that presence of objects changes over medium spatial scales of tens of kilometres. This is in accordance with the gross observations summarised in figure 2-6, demonstrating that some fields were characterised by the presence of numerous objects and that other fields were free from any infrastructure.

Table 2-3. Results of the spatial statistical. Significant *p*-value are highlighted in bold.

	F	Pr(>F)	Proportion of variance explained by model
Variable selection step 1: spatial eigenvector selection			
Full spatial RDA model with all 15 MEM eigenvectors	22.494	**0.001**	0.1224
Restricted RDA model with 3 most significant MEM eigenvectors (MEM2, MEM1 and MEM15)	95.375	**0.001**	0.1053
Variable selection step 2: overall variable selection			
Full RDA model with 3 MEM eigenvectors and all environmental and anthropogenic variables	19.164	**0.001**	0.1761
Restricted RDA model with 8 most significant variables (MEM1, substrate, object, Ruggedness, Fishing effort, distance to well, MEM15 and temperature)	31.449	**0.001**	0.1569
Final model			
pRDA model with significant environmental and anthropogenic variables as constraints and significant spatial variables as condition	40.575	**0.001**	Constraint = 0.1170 Condition = 0.0547

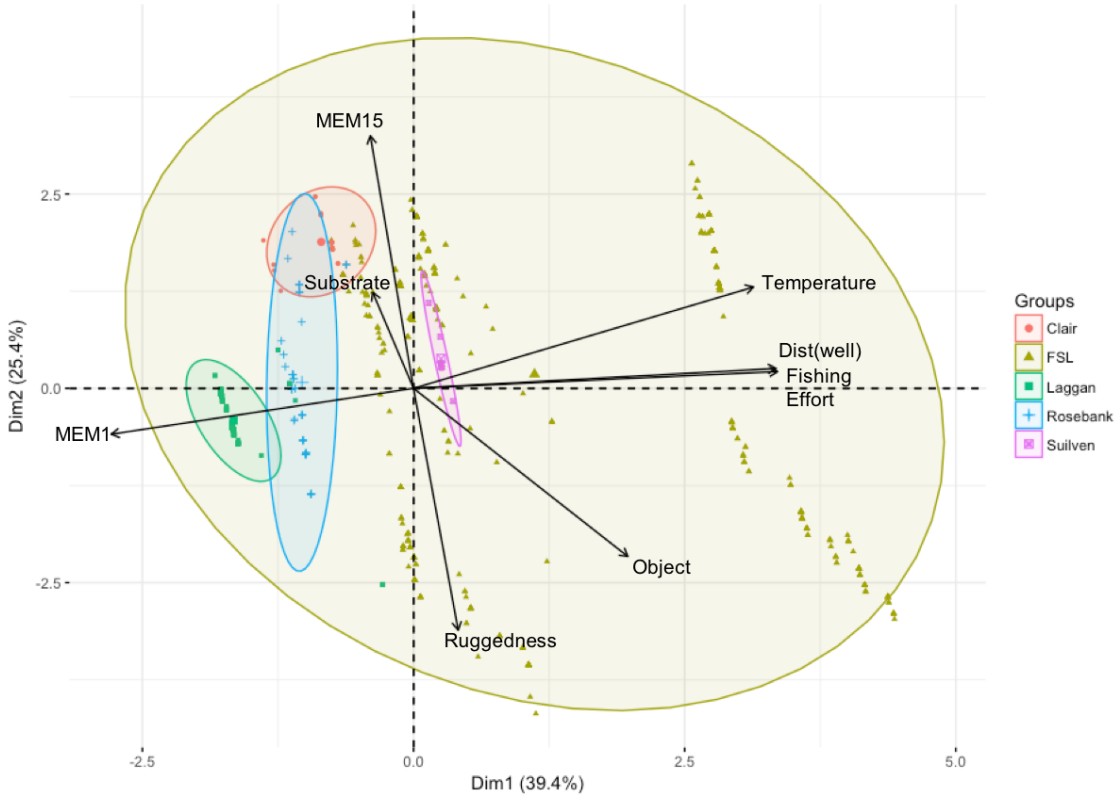

Figure 2-7. Biplot of the principal component analysis (PCA) model. Each data point represents a still image. The patterns in data points are due to the differences in data resolution. Ellipses have been drawn around data points from each site available in this analysis.

2.4.4. Impacts of environmental and anthropogenic factors on megafaunal community composition in the Faroe-Shetland channel

A pRDA was performed to determine the impact of environmental and anthropogenic variables on the spatial distribution of the FSC megafauna. The pRDA model, constructed using all data points available in this study, was statistically significant ($F= 40.575$, p-value<0.001). The pRDA model captured in total 17.2% of the OTU variability within the large study area (Table 2-3). Although the level of variability explained by the pRDA model can seem low, it is important to take into account the size of the study area (across oil fields), the number of OTUs considered as well as the resolution of the environmental and anthropogenic variables available. The constraint term applied in the model (eight explanatory variables selected above) accounted for 11.7% of the megafauna composition variability while the condition term (two spatial eigenvectors) accounted for the remaining 5.5%. Substrate type and ruggedness constituted the main factors influencing megafaunal community composition in the FSC (Figure 2-8). Temperature, object presence, fishing effort and distance to the closest well also appeared to impact megafauna composition but to a lesser extent (Figure 2-8).

Differences between OTUs could also be observed when considering the pRDA biplot (Figure 2-8), with the available OTUs being strongly influenced by specific environmental variables and to a lesser extent, by anthropogenic factors. Sponge presence appeared mainly driven by substrate type and most morphotypes were associated with coarser substrates (Figure 2-8). Presence of other sessile benthic megafauna such as Crinoida, Cirripeda and Alcyonacea seemed also mainly influenced by substrate type and ruggedness. The distribution of sponge morphotypes and other sessile fauna were also clearly impacted by fishing effort with all sessile fauna being associated with areas of low level of fishing activities. Mobile benthic megafauna such as polychaetes, ophiuroids, gastropods, holothurians and echinoids seemed strongly impacted by ruggedness only. The two fish OTUs appear to be associated with larger objects but away from the drilling wells.

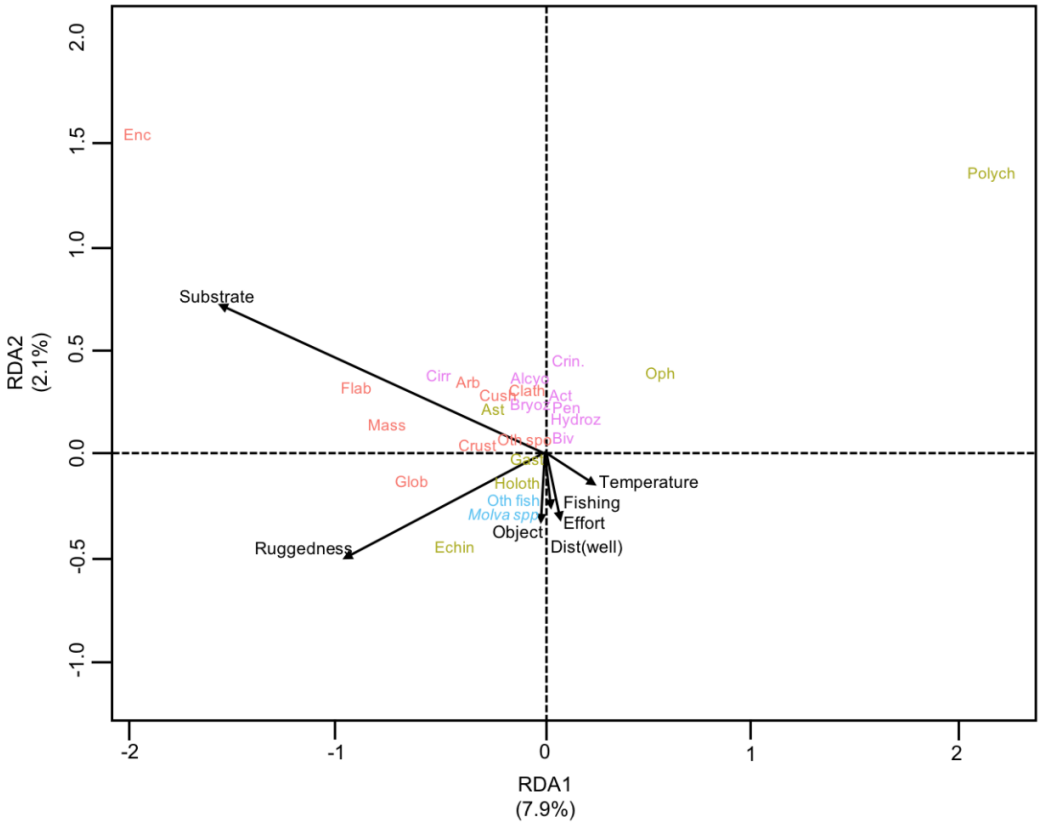

Figure 2-8. Biplot of partial redundancy analysis (pRDA) model. For clarity, sponge OTUs are highlighted in orange, other sessile megafauna in pink, fish OTUs in blue and other mobile megafauna in green.

2.5. Discussion

2.5.1. Megafaunal diversity in the Faroe-Shetland channel

Overall megafauna composition recorded in this chapter are in accordance with previous studies (Axelsson, 2003; Jones *et al.*, 2007a; Howell *et al.*, 2007). It had been

reported that Cnidaria, Polychaeta, Porifera, Enteropneusta, Mollusca and Crustacea together represented 90% of the megafauna present in the FSC (Axelsson, 2003). Fish (blackbelly rosefish *Helicolenus dactylopterus*), squat lobsters, urchins, sea stars, anemones, holothurians, brittle stars as well as sponges were also described as often encountered in the FSC (Howell *et al.*, 2007). Jones *et al.* (2007a) reported high abundances in Annelida and Cnidaria across the FSC with other Phyla including Anthropoda, Echinodermata and Porifera detected at high density at specific stations. In this study, Porifera, Polychaeta, Crustacea, Echinoidea and Ophiruidea were the most recorded phyla. Eight sponge morphotypes were used in this analysis, highlighting the diversity of sponges present in the FSC. This is also in accordance with previous publications (Axelsson, 2003; Howell *et al.*, 2007).

2.5.2. Role of substrate characteristics on megafaunal distribution in the Faroe-Shetland Channel

In this study, variables defining seabed characteristics such as substrate type and ruggedness seemed to play a major role in driving benthic megafauna composition. Substrate type along with depth, is known to be an important factor driving deep-sea benthic community composition (Howell, 2010; Lacharité and Metaxas, 2018). Data presented in this study additionally showed that substrate type had a stronger impact on sessile fauna including Porifera, Cirripedia, Alcyonacea and Crinoidea, with most being associated with coarser substrate.

The predominant substrate types recorded in this chapter were gravel/cobble and sand, which is in accordance with previous studies in the FSC (Bett, 2001; Jones *et al.*, 2007a; Howell *et al.*, 2007). Seabed morphology in the FSC was thought to be created by sediments deposited in the last glacial age and then modified during the Holocene (Masson, 2001). Features from the last glacial age such as iceberg plough marks characterised by parallel bands of cobbles separated by softer sediment substrate, have been described to have a strong impact on substrate type composition in the FSC (Bett, 2001; Axelsson, 2003; Howell *et al.*, 2007). No clear plough mark structures could be distinguished in our study, but this is in accordance with previous video transect surveys and could be a result of plough marks being degraded from local hydrodynamic regime (Howell *et al.*, 2007). Substrate type and ruggedness was found to change over smaller spatial scales in this study. Finding differences in substrate characteristics between close locations could be related to the mentioned post-depositional modification to the sediments.

2.5.3. Consideration on temperature and water masses effects on megafaunal distribution in the Faroe-Shetland channel

Although substrate characteristics were detected as the most significant factors influencing megafaunal benthic distribution in the FSC, temperature also played a role in controlling benthic megafauna distribution. The FSC is characterised by complex water circulations as five water masses with different salinities, temperatures and nutrient concentrations flows through the channel (Hansen and Østerhus, 2000). The North Atlantic Water and the Modified North Atlantic Water are present in the upper layers of the channel travelling in a northeast direction towards the Arctic Seas while the Modified East Icelandic Water, Norwegian Sea Arctic Intermediate Water and Norwegian Sea Deep Water have been identified moving below, in a southwest direction towards the North Atlantic (Hansen and Østerhus, 2000). Generally, water temperature on the Shetland side of the FSC can vary from 0°C or lower below 700 m depth to over 10°C above 100 m depth (Berx *et al.*, 2013). Temperature considered in our model was shown to vary over large scales of dozens of kilometres. This is in accordance with the large-scale mixing patterns, discussed above, known to characterise the FSC.

Seasonal mixing patterns of the five water masses present in the FSC are complex, and some quantification of their mixing has only recently been achieved (Berx *et al.*, 2013; McKenna *et al.*, 2016). Furthermore, topographic eddies have also been identified in the deeper part of the channel, altering the deep currents pathway and the distribution of the deeper water masses (Broadbridge and Toumi, 2015). Internal tides and near-bed waves have been detected in the southeastern flank of the FSC leading to higher levels of mixing on the slope (Hall *et al.*, 2011). Formations of cyclonic eddies and meanders mixing the North Atlantic Water and Modified North Atlantic Water have also been observed (Sherwin *et al.*, 2006). Through this complex mixing regime, benthic organisms present in the FSC can therefore be exposed to great changes in temperature of up to 7°C in an hour (Bett, 2001). Ostur sponge grounds as described by Bett (2001) experience, at least occasionally, sub-zero temperatures while the ostur communities described by Klitgaard *et al.* (1997) around the Faroes thrive under water temperatures higher than 5ºC.

Investigation into the elemental composition of dissolved organic matter (DOM) in the FSC revealed that mesopelagic water of the channel transported N-rich DOM from arctic surface water (Kramer *et al.*, 2005). In the Arctic, terrigenous inputs from rivers

and seasonally highly productive surface water enrich water masses in dissolved organic nitrogen (Letscher *et al.,* 2013). Deep-sea sponges rely on DOM and particulate organic matter (POM) for their feeding (Yahel *et al.*, 2007; Rix *et al.*, 2016; Kazanidis *et al.*, 2018). In our study, neap tide and spring tide seabed stress significantly influenced the benthic community composition. Bed stress can be defined as the pressure exerted by water movement at the bottom. Intense water movement at the substrate promotes the resuspension of POM which is beneficial for filter-feeders such as sponges. It could therefore be hypothesised that sponges and other filter-feeders present in the FSC in areas with coarse substrate and benefit from chronic inputs of organic matter through upwelling of the deep Arctic waters.

2.5.4. Significance of anthropogenic activities in the Faroe-Shetland channel

The pRDA model considered in this chapter took into account several anthropogenic factors reflecting the multiple activities taking place in the FSC. Although environmental variables such as substrate characteristics and temperature had the strongest influence on benthic community composition, it is clear that both fishery and hydrocarbon production activities also impact local megafauna. Both activities seem to have impacts on sessile and motile fauna. Diverse communities of sessile epifauna such as crinoids, soft corals, and clathrate sponges and the commercially important fish *Molva sp* were recorded closer to oil and gas infrastructures (but away from drilling wells).

Commercial deep-water fishing is known to have occurred in the FSC (Bullough *et al.*, 1998; Bett, 2001) and both trawl marks as well as fishing gear (ropes) were detected in this study. Furthermore, commercially important fish species including *Molva sp* (Ling) were also recorded. Fishing effort varied over large spatial scales. This could potentially be a result of the coarse resolution of the available data. Furthermore, although fishing effort has a significant impact on megafauna spatial distribution, it does not appear to be as strong as oil and gas related variables. The presence of trawl marks was also not retained in the models presented in this chapter as it was not statistically significant. The impact of deep-water trawling on deep-sea benthic communities in our study could have been reduced owing to the origin of the data. Data from the environmental surveys conducted by oil and gas companies were used here. These were targeted around oil and gas infrastructures and fishing activities are not allowed within 500 m of oil and gas infrastructures. However, data were also collected in areas of interest to the oil and gas industry; in fact, over 25% of the still images analysed in this study were recorded at more than 1 km away from the closest well and pipeline. Another hypothesis to explain the

lower influence from fishery activities in our analysis is that fishery in the FSC occur at greater depths than 500 m (Bullough *et al.*, 1998). However, 38% images available in our study were recorded at depth greater than 500 m, within fishing depth range. The impacts on deep-sea fishing on benthic ecosystems have recently been documented in a review by Clark *et al.* (2016) and include changes to seabed topography and substrate, physical damage to the benthic fauna leading to changes in community characteristics (Clark *et al.*, 2016). The current proposed management plan from Marine Scotland for the FSC NCMPA include restriction zones for all demersal gears along the south-eastern border of the NCMPA and for demersal trawls along the north-western border of the NCMPA with a fishing corridor present between the two areas (Marine Scotland, 2017ab).

The impact of oil and gas activities in the FSC has previously been studied in several publications. As discussed in the first chapter, physical disturbance from offshore drilling led to a decrease in diversity in terms of species richness at several sites within the FSC (Jones *et al.*, 2006, 2007b). Overall megafaunal richness increased with distance from the disturbance, with the highest impacts on the benthic megafauna visible within 50 m from the drilling site (Jones *et al.*, 2006, 2007b). A follow-up study published in 2012 showed that the megafauna had only partially recovered between 3 and 10 years after the drilling had occurred (Jones *et al.*, 2012). Here, distance to the closest well was a significant factor impacting megafauna composition, which seems to be in agreement with the studies discussed above. Distance to the closest well changed across oil fields, highlighting the fact that the fields considered here were at different developmental stages. This is an important finding as it also means that the impact of distance to the closest well would not have been detected if considering individual sites (i.e. oil fields). Only by sampling datasets covering several sites can this strong effect of offshore oil and gas activities be determined. As faunal recovery from drilling in the deep sea is often slow (Jones *et al.*, 2012; Henry *et al.*, 2017), only considering distance to well rather than time of drilling in the analysis appears to be adequate. It can hypothesized here that the presence of drill cuttings close to wells in the FSC is driving megafauna composition changes towards environmentally suitable areas away from wells, but close to other infrastructure. This analysis shows that consideration of spatial scales is important when aiming at understanding how environmental factors and anthropogenic activities impact the spatial distribution of species. Several studies have highlighted the significance of spatial scale in deep-sea ecology studies (Henry *et al.*, 2013; Ingels and Vanreusel, 2013; De Leo *et al.,* 2014). However, no previous work has applied dbMEM analysis to

determine the scale of impact of multiple anthropogenic activities on deep-sea benthic communities.

2.5.5. Potential considerations for the oil and gas industry

In this study we found that presence of fish OTUs including commercially-important *Molva spp* was linked with the presence of larger oil infrastructures such as pipelines and manifolds furthest away from the wells. The association of fish species with oil production infrastructures in use or after decommissioning has previously been documented (Seaman *et al.*, 1989). Oil platforms off California were shown to host the most productive fish habitats per unit area of seafloor of any marine ecosystem (Claisse *et al.*, 2014), with productivity values varying greatly from one rig to another (Fowler *et al.*, 2015). Furthermore, the presence of sessile benthic organisms on oil rigs has also been described in the literature, including cold-water corals (Gass and Roberts, 2006, 2010; Kolian *et al.*, 2017; Gormley *et al.*, 2018). It has been suggested that leaving decommissioned structures in place could be, in some instances, beneficial for deep-sea benthic ecosystems (Kolian *et al.*, 2017; Fowler *et al.,* 2018). In addition to the economic and safety benefits, partial removal of assets could limit physical disturbances to the seabed and damage deep-water ecosystems (Fowler *et al.*, 2014; Techera and Chandler, 2015). Rigs-to-reefs projects have been developed around this idea (Techera and Chandler, 2015; Reggio and Villere 1987). As mentioned in chapter 1, decommissioning of oil and gas industry infrastructure has not yet taken place within known deep-sea sponge grounds and so potential impacts of decommissioning on the local megafaunal community are for the moment unknown. Since several OTUs in this study are found in association with larger infrastructures in the FSC, decommissioning considerations for the FSC will have to take into account the sensitivity of these OTUs to disturbances.

2.6. Conclusion

The study presented in this chapter combined datasets from both industry and academic sources. The aims of the chapter were:

(1) To provide a better understanding of the environmental factors influencing the spatial distribution of the different sponge morphotypes and associated megafauna community present in the Faroe-Shetland channel.

(2) To analyse the impact of anthropogenic activities (fishing and hydrocarbon production) on benthic megafauna in the Faroe-Shetland channel.

The spatial statistical analysis conducted in this chapter showed that scale of observation needs to be taking into account when considering the environmental and anthropogenic

impacts over a large sampling area. Bottom temperature and fisheries effort both exerted strong control over species assembly at the largest spatial scales in the FSC (across the whole region). Impacts from oil and gas activities (distance to well) and finer-grained shifts in environmental factors such as substrate and rugosity were detected at smaller scales (within a hydrocarbon field). Overall sessile fauna were found associated with coarse and rugose substrate in areas with lower fishing activities while motile fauna (especially fishes) seemed to accumulate around oil and gas infrastructures away from well sites.

As reviewed in chapter 1, oil spills constitute a considerable risk associated with offshore oil and gas activities and significant knowledge gaps on the resilience of sponges to exposure to contaminated seawater and sediments persists. The next three chapters of this thesis will present experimental work undertaken to fill these knowledge gaps.

Chapter 3 Impacts of Crude Oil and Dispersed Crude Oil Contaminated Seawater on Sponge *Halichondria panicea* at Physiological, Molecular and Histological Levels

3.1. Overview

The purpose of this chapter is to present the experimental work conducted on *Halichondria panicea* with hydrocarbon-contaminated seawater. This chapter has been adapted to a manuscript, which will soon be submitted to the scientific journal Environmental Science and Technology (see appendix A).

3.2. Introduction

As highlighted in chapter 1, little is known about the capacity of sponges to cope with environmental stressors such as chemical pollution. Sponges high filtration rates mean that they can bioaccumulate a range of chemicals including polyaromatic hydrocarbons (PAHs) (Batista *et al.*, 2013; Mahaut *et al.*, 2013), polychlorinated bisphenyls (Gentric *et al.*, 2016) and metals (Olesen and Weeks, 1994; Berthet *et al.*, 2005). While this has led to research on sponges for biomonitoring purposes (Châtel *et al.*, 2011; Batista *et al.*, 2013; Mahaut *et al.*, 2013; Gentric *et al.*, 2016), the biological impacts of contaminants in sponges, including petroleum hydrocarbons, have remained poorly understood. PAHs have been shown to inhibit sponge *Crambe crambe* larvae settlement (Cebrian and Uriz, 2007). Furthermore, exposure to the PAH Benzo[a]Pyrene (BaP) induced DNA damage in *Tethya lyncurium* (Zahn *et al.*, 1981, 1983). Activation of the mitogen-activated protein kinases (MAPK) cell signalling pathway has also been detected in *Suberites domuncula* exposed to diesel contaminated seawater (Châtel *et al.*, 2011). However, further studies are needed to better understand the biological impacts in sponges of petroleum hydrocarbons that can occur, at high concentrations, during an accidental spill.

Although spills are rare events, they can have significant short and long-term biological effects depending on the organisms and ecosystems impacted (Shigenaka, 2011). In shallow-water environment, sublethal short-term repercussions include changes to gene expression patterns (Hamdoun *et al.*, 2002; Boutet *et al.*, 2004; Hook and Osborn, 2012; Jenny *et al.*, 2016), elevated oxidative or physiological stress (Kennedy and Farrell, 2005; Lyons *et al.*, 2011), decrease in immune functions (Hannam *et al.*, 2010) and larval disruptions (Bellas *et al.*, 2013; Negri *et al.*, 2016; Stefansson *et al.*, 2016). Long-term impacts include sustained slow growth and reproduction rates (Suchanek, 1993; Culbertson *et al.*, 2008), decreased diversity (Suchanek, 1993) and community structure anomalies (Kingston, 1999). The chemical dispersants sometimes used to remediate oil spills can also themselves be toxic (McKeown and March, 1978; Epstein *et al.*, 2000; Edwards *et al.*, 2003; Venn *et al.*, 2009) or increase the toxicity of spilled oil mixture

(Singer *et al.*, 1998; Ramachandran *et al.*, 2004; Couillard *et al.*, 2005; Zhang *et al.*, 2013). Furthermore, dispersants can persist in the environment, raising concerns for long-term biological impacts (White *et al.*, 2014).

To study the impact of oil and dispersants on sponges in a laboratory environment, water accommodated crude oil fractions (WAFs) and chemically enhanced WAFs (CEWAFs) should be used. WAF is defined as a laboratory solution produced by mixing crude oil and seawater with limited energy (slow stirring speed without the formation of a vortex) (Aurand and Coelho, 2005). The resulting solution should be free of visible oil droplets (Aurand and Coelho, 2005). CEWAF is defined as a laboratory solution obtained by mixing crude oil, chemical dispersant and seawater with medium energy (medium stirring speed with small vortex formation) (Aurand and Coelho, 2005). The resulting solution can contain small oil droplets in suspension (Aurand and Coelho, 2005). How to best prepare solutions of WAF and CEWAF is under discussion in the scientific literature and several protocols have emerged (Redman and Parkerton, 2015). Many studies, however, have focussed on understanding the effect of WAF and CEWAF on model organisms such as mysids, topsmelts, abalones (Singer *et al.*, 1998), microalgae (Wolfe *et al.*, 1999), barnacles (Greco *et al.*, 2006), nematodes (Zhang *et al.*, 2013), shrimps (Rodd *et al.*, 2014) or zebrafish (Philibert *et al.*, 2016). No study to date has investigated the impact of crude oil WAF and CEWAF on sponges.

Amongst temperate shallow-water sponges, *Halichondria panicea* is a species that has been more widely studied. Early publications have described *H. panicea* seasonal growth rate, silica uptake, reproduction and physiology (Barthel, 1986, 1988; Riisgard *et al.*, 1993; Thomassen and Riisgaard, 1995; Frøhlich and Barthel, 1997; Schönberg and Barthel, 1997; Osinga *et al.*, 1999) while later work focussed on the bacterial community associated with this sponge species (Althoff *et al.*, 1998; Wichels *et al.*, 2006; Schneemann *et al.*, 2010). The relationship between *H. panicea* and its environment and how the sponge interacts with other organisms such as mussels and nudibranchs have also been investigated (Knowlton and Highsmith, 2005; Khalaman and Komendantov, 2016; Khalaman *et al.*, 2017). More recently *H. panicea*'s diet, filtration rate and dynamic osculum behaviour have also been researched (Riisgård *et al.*, 2016; Kumala *et al.*, 2017). Some information on the sponge tissue organisation is also available (Eerkes-Medrano *et al.*, 2015). Furthermore, the impact of cadmium pollution on *H. panicea* physiology has been studied (Olesen and Weeks, 1994). Widely present in Scotland at intertidal depth

(Ackers *et al.*, 2007), *H. panicea* is easy to sample and therefore constitutes an ideal model sponge species to use in experimental work.

The purpose of the work presented in this chapter is to determine the biological impacts of WAF and CEWAF on the marine shallow-water sponge *H. panicea*. Specifically, the effects of WAF and CEWAF on *H. panicea* physiology (respiration and clearance rate), gene expression (transcriptomic and quantitative PCR) and tissue organisation (histology) were investigated. Considerations on how the results presented in this thesis on *H. panicea* can be expanded to other sponge species (shallow and deep-sea sponges) is discussed in chapter 6. Preliminary work on *H. panicea* on aquarium husbandry and development of the experimental set-up used in this chapter, is presented in appendix B. Finally results from a comparative study performed on another shallow-water sponge, *Myxilla sp*, collected at 10m depth by SCUBA from Loch Creran can be found in appendix C.

3.3. Material and methods

3.3.1. Sample collection

H. panicea samples were collected at Coldingham bay, located 80 km to the south of Edinburgh, Scotland. *H. panicea* can easily be found in the bay, at low tide, colonising rocks underneath kelps where it grows in a yellow-green encrusting/cushion morphotype (Figure 3-1A). Sponges were carefully removed with a scalpel from the rocks and placed into sampling bags filled with freshly collected seawater. This sampling method allowed for samples of good size (2-3mm in thickness and 4-5cm across) to be retrieved from the rock (Figure 3-1BC).

Upon return to the University of Edinburgh, samples were kept in retention tanks in a cold-room at 10° C, for up to three weeks, prior to the experiments (see appendix B for further details on aquarium husbandry). Eight litres of surface seawater were also collected at Coldingham bay to be used for the preparation of treatment solutions. Upon return to the University of Edinburgh, the seawater samples were also kept in a cold-room at 10° C and aeration was provided until the samples were needed. No filtration was conducted on the seawater samples to allow the WAF and CEWAF solutions to reflect as far as possible the conditions of an oil spill in the environment.

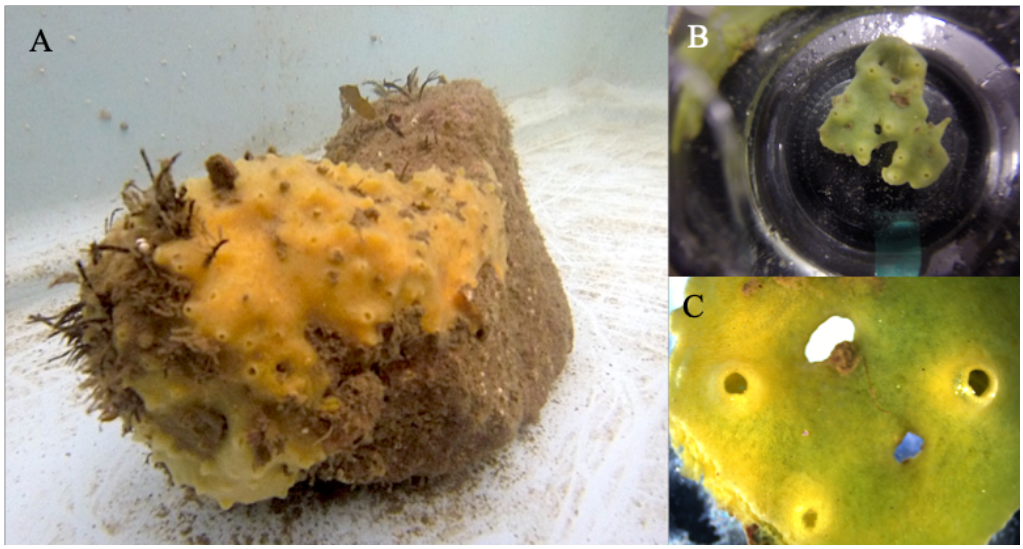

Figure 3-1. Examples of *Halichondria panicea* collected at Coldingham bay. (A) *H. panicea* growing on a rock in a tank at St Abbs marine station. (B) *H. panicea* sample in incubation chamber. (C) *H. panicea* control sample at the end of preliminary experiment under binocular microscope.

3.3.2. Experimental set-up

Limitations

In order to conduct experiments exposing sponges to crude oil, an experimental design was selected, based on the following criteria:

(1) The experimental apparatus should limit the use of plastics such as PolyVinyl Chloride and Polyurethane often used for aquarium tubing as hydrocarbons interact with these plastics and attach to their surfaces

(2) The experimental apparatus needs to limit the quantity of contaminated seawater produced through each experimental run so that only the exact volume of contaminated seawater is produced for each experimental run

(3) The experimental apparatus should contain all produced contaminated water throughout the experimental run and limit air contamination from volatile hydrocarbons.

Based on these criteria, a flow-through experimental design where contaminated seawater is pumped through a series of glass and PolyTetraFluoroEthylene (PTFE) incubation chambers was selected.

Incubation chambers

To conduct exposure experiment with *H. panicea*, bespoke incubation chambers in a flow-through experimental apparatus were used. In total, 16 chambers of 750 mL of volume were manufactured. The chambers were made of a beaker of 80 mm in diameter in tempered glass while the lid was constructed of PTFE (Figure 3-2A). Each subset of

four incubation chambers could be placed onto a holding plate and the lids could be locked onto the chamber by tightening a metal panel with bolts placed above the lid (Figure 3-2B). A water-proof magnetic rotor column was attached to the centre of each holding plate. This magnetic rotor column enabled the magnetic stirrers attached to the lids of the four chambers placed around the column to move (Figure 3-2B). Each lid was also equipped with a Presens sensor spot (Presens Precision Sensing GmbH, Germany) enabling direct respiration measurement without the need to transfer the sponge into respiration chambers as well as inflow and outflow valves. The inflow valves were fitted with inner PTFE tubing so that inflow water entered from the lower half of the chamber (Figure 3-2AB).

Figure 3-2. Incubation chambers and experimental apparatus. (A) Incubation chamber with (1) locking system, (2) inflow PTFE tube and (3) magnetic stirrer. (B) Holding plate with four incubation chambers. At the centre of the plate, a waterproof magnetic rotor column controls the magnetic stirrers in the four adjacent chambers. (C) Experimental apparatus with (1) Duran® bottles containing the treatment solutions, (2) Marine Color™ peristaltic pumps, (3) incubation chambers on holding plates and (4) used seawater collection bucket.

Chambers were connected through PTFE tubing to three six-channel Marine Color™ peristaltic pumps (Figure 3-2C). Each channel of the peristaltic pumps was itself connected back to a one litre Duran® bottle containing the relevant treatment solution (Figure 3-2C). Air bubbling was provided into the Duran® bottle through PVC tubing

connected to glass Pasteur pipettes so that the treatment solutions were only in contact with the glass pipette. Flow was set at 750 mL per 24 h to allow replenish the chamber every day.

Experimental design

To investigate the impact of WAF and CEWAF on *H. panicea* at physiological, histological and molecular levels, two types of experiments were conducted: a single concentration experiment and two dose-response experiments. The experimental set-up described in chapter 3 was used to perform both experiment types. In the single concentration experiment, sponge samples were exposed to WAF (1 g/L of oil), CEWAF (1 g/L of oil with dispersant) and BaP (10 ppb as described in Zahn *et al.*, 1983) dissolved in DMSO for 48 h (three replicates per treatment). Sponge samples were also kept in two control conditions: seawater and DMSO (three replicates per condition). Sponges were placed in the incubation chambers described above and left to acclimatise for 48 h before starting the exposure. Sponges were then exposed for 48 h to the relevant treatment solution. Clean seawater was pumped back into the chambers for another 48-hour period before the end of the experiment. Dose-response experiments were also conducted during which sponges were exposed to increasing nominal concentrations of crude oil (WAF dose-response) or crude oil and dispersants (CEWAF dose-response) for 48h. For both treatments, the nominal oil loading ranged from 0.01 g/L to 10.0 g/L of oil. Sponges were placed in their chambers and left to acclimatise for 48 h before exposure, as for the single concentration experiment. The sponges were then exposed to their treatment for 48 h after which the experiments were terminated.

3.3.3. Treatment solutions

Crude oil and dispersant used

In all experiments presented in this thesis, Schiehallion crude oil (BP) and dispersant Slickgone NS (Dasic International) were used. Schiehallon crude oil is produced at Schiehallion oil field in the Faroe-Shetland channel and was provided by BP. The crude oil is characterised by an American Petroleum Institute gravity of 25.2, a sulphur content of 0.46 % and a viscosity of 67 centistokes (cST) at 20°C (BP, 2017). Slickgone NS, provided by the Oil Spill Response Limited, is one of the dispersants approved for use by the United-Kingdom Marine Management Organisation and is listed for potential use in the Faroe-Shetland channel in the case of a spill (BP, 2014; Marine Management Organisation, 2018).

Treatment solution preparation

The chemical response to oil spills ecological research forum (CROSERF) protocol, which allows for the preparation of WAF and CEWAF at different nominal oil loadings, was followed (Aurand and Coelho, 2005). For the single concentration experiment, WAF at 1 g/L of oil loading and CEWAF at 1 g/L of oil loading were prepared. For the dose-response experiments, WAFs and CEWAFs at 0.01, 0.03, 0.05, 0.1, 0.5, 1.0, 2.5, 3.5, 5, 7.5 and 10.0 g/L oil loadings were prepared. The amount of crude oil added to seawater was first weighed. A glass syringe was used to sample and transfer the crude oil to a Duran® bottle filled with one litre of seawater. The glass syringe was weighed before and after the transfer to determine the exact weight of oil added to the seawater. When preparing CEWAF, dispersant was applied after the addition of the oil to the seawater at a volume ratio of 1:10 as advised by the manufacturers (Dasic International OSD Limited, 2018). The mixture was then mixed for 18 hours using a magnetic multi-plate stirrer at low (for WAF) or medium speed (for CEWAF). At the end of the 18 h mixing time, solutions were left to settle for three hours and the water fraction was then carefully removed and placed into a clean one litre Duran® bottle to be used in the experiments.

BaP (positive control) and dimethyl sulfoxide (DMSO) (negative control along with seawater) solutions were also prepared for the single concentration experiment. A stock solution of BaP in DMSO at a concentration of 0.1 g/L was first prepared. For the BaP treatment solution, 100 µL of stock solution was then added to 999.9 mL of seawater prior to the use in the experiment (no mixing time was allowed for this treatment). The same volume of DMSO only was also added to seawater in the DMSO only treatment.

Analytical chemistry

To characterise the conditions *H. panicea* samples were exposed to, WAF and CEWAF samples from the dose-response experiments were analysed and the concentrations of 16 PAHs were determined. Gas chromatography mass spectrometry (GC-MS) analysis of water samples was conducted in collaboration with Dr C. Montagner (University of Campinas, Brasil). Collection of 100 mL water samples occurred at the end of each exposure. Molecular grade dichloromethane (10 mL) was added to each sample before further analysis could be carried out. A separating funnel enabled the separation of the dichloromethane phase (with the hydrocarbons) from the aqueous phase. Each aqueous phase was re-extracted twice. A rotary evaporator was then used to slowly remove the solvent material before analysis.

After sample preparation, the extracts were diluted in a 0.5 mL of dichloromethane. The chromatographic analysis of the 16 polyaromatic hydrocarbons (PAHs) was carried out on a GC-MS equipment (Shimadzu, model QP-5050 A) based on the EPA 8270 method using a capillary column DB-5MS (J&W Scientific): 30 m x 0.25 mm ID x 0.25 µm film thickness. A 1 µL aliquot of the final extract was injected in the split mode. Data acquisition was performed in the SIM mode. Each PAH was individually quantified using a (1-1000 µg L^{-1}) calibration curve obtained after the appropriate dilution of an analytical standard solution (48743 Supelco, EPA 610 PAH Mixture). The limit of quantification varied between 1 and 10 µg L^{-1} and the correlation coefficients of each individual PAH analytical curve were above 0.99.

3.3.4. Physiological endpoints

Sponge volume determination

The volume of each sponge sample, to be used for the normalisation of the respiration and clearance rate, was determined at the end of each experiment. The thickness of the sample was measured directly with a calliper. A photography was then taken of each sample and the freely available software Fiji was used to determine the surface area of the sample (Schindelin *et al.,* 2012). Surface area was then multiplied by the thickness to determine the sponge volume. For this technique to be precise, the thickness of the tissue needs to be homogenous throughout the sample. *H. panicea* present at Coldingham Bay are of an encrusting morphotype, with little variation in the layer thickness. Standardisation to the volume is therefore here applicable and was chosen even though standardisation to other parameters, such as carbon content, could have also been considered.

Respiration rate measurements

Respiration rate was determined by analysing the decrease in O_2 over time in each chamber. Concentration of O_2 in each chamber was measured every 15 seconds during two hours using Presens sensor spots connected to Oxy-4 optodes (Presens Precision Sensing GmbH, Germany). A two-hour time period was enough to detect a significant change in O_2 concentration in the respiration chamber. Stirring in the chambers was kept active during the measurement time but the inflow and outflow were closed so no fresh input of seawater was added. To account for microbial respiration in the seawater, blank measurement of respiration in seawater and treatment solutions were also determined in

empty chambers. $\%O_2$ was converted to µmol/L using the R package presens (Birk, 2016). Sponge respiration rate were then determined using the following formula:

$$Respiration\ rate = (Resp_{Chamber} - Resp_{blank})/V_{sponge}$$

where $Resp_{chamber}$ is the respiration rate determined in the chamber with a sponge, $Resp_{blank}$ is the respiration rate determined in the blank chamber without a sponge and V_{sponge} the volume of sponge tissue (determined at the end of the experiment).

Clearance rate measurements

Clearance rate is an estimation of the amount of water filtered by the sponge per unit of time and per unit of sponge volume and can be used as a proxy for filtration rate. To determine clearance rate, *Isochrysis* Instant Algea® (Reed Mariculture, California) diluted solution was added to each chamber and the sponges were left to filter for two hours. Stirring in the chambers was kept active during the measurement time but the inflow and outflow were closed so no fresh input of seawater was added. Water samples were collected every 20 minutes and algae cell concentrations were determined through total absorbance measurements. Clearance rates for each sample was calculated as follows (De Goeij *et al.*, 2008a):

$$Clearance\ rate = (\left(\frac{V_{water}}{t}\right) \ln\left(\frac{C_0}{C_t}\right))/V_{sponge}$$

where V_{water} is the volume of water in the chambers, t the time of incubation, C_0 and C_t the initial and final concentration of algae in the chamber and V_{sponge} the volume of sponge tissue.

Statistical analysis of physiological endpoints

To determine if treatment influenced respiration and clearance rates during and after exposure in our single concentration experiment, a repeated-measure Analysis of Variance (ANOVA) was carried out with Rstudio (R Core Team, 2017). Using the R package lme4 (Bates *et al.,* 2015), a linear mixed-effect model was fitted to each data set with treatment, time, treatment*time as fixed explanatory factors and individual sponge as a random effect. Residual normal distribution and sphericity was checked graphically. To determine the effect of increasing oil loading in WAF and CEWAF solutions on respiration and clearance rate (dose-response experiments), a dose-response analysis was performed using the package drc (Ritz *et al.,* 2015). A dose-response model was fitted to each dataset (respiration and clearance) using a Weibull 1 three parameter (upper asymptotic limit, slope and ED50) function with treatment as a grouping factor. As controls from WAF and CEWAF experiments did not statistically differ from each other,

the model was constrained to a single asymptotic limit while slope and ED50 were estimated independently for each treatment. A lack-of-fit test and no effect test were then carried out to determine if the models were significant. Further comparison of significant parameters between treatments was also carried out using the package drc (Ritz *et al.,* 2015).

3.3.5. Molecular endpoints

Tissue sampling

Tissue samples were collected during the experiments to allow for molecular work to be conducted. About 3 mm^3 of tissue was cut from individual sponge samples with a sterile scalpel and thoroughly rinsed with MilliQ™ water to remove any outer debris and seawater. Samples were then preserved in RNAlater® (Sigma-Aldrich, UK) and placed at -20° C.

RNA extractions

Total RNA was extracted from sponge tissue samples using Qiagen (UK) RNAeasy® extraction kits following the manufacturer's instruction. Small sections of sample (about 1 mm^3) were homogenized using a MSE SoniPrep (UK) sonicator (5s burst at level 4) and lysed using the provide lysing buffer. DNA was removed from the RNA extractions with Qiagen RNase-Free DNase sets. At the end of the protocol, RNA was eluded into 30 µL of DNA/RNA free sterile water. RNA quality and quantity were then assessed by spectrophotometer by NanoDrop™ (NanoDrop™ 2000, ThermoFisher Scientific (UK)). Only RNA samples with a 260/230 and 260/280 ratio of 1.8-2.2 were used in further analysis. RNA samples were stored in -80° C until sequencing or reverse transcription was performed.

Reverse Transcriptase PCR (RT-PCR)

Reverse transcriptase PCR (RT-PCR) was performed, to obtain cDNA, using nanoScript™ 2 (UK) Reverse Transcription kits following manufacturer's instructions. RNA samples were diluted to a concentration of 100 ng/µL prior to the RT-PCR. Each reaction contained 9.0 µL of RNA template, 1.0 µL of RT primers, 5.0 µL of nanoScript2 4X buffer, 1.0uL of dNTP mix, 3.0 µL of water and 1.0 µL of nanoScript2 enzyme. The annealing step of the RT-PCR was conducted at 65°C for 5min. After that, the extension step was carried out during 30 cycles of 42°C for 20min followed by 75°C for 10min. cDNA samples were stored at -20°C until qPCR was performed. Both RT-PCR steps were

conducted on an Applied Biosystems Veriti 96 well thermocycler (ThermoFisher Scientific, UK).

Transcriptomics

As primers available in the literature did not yield any relevant amplification in *H. panicea*, transcriptomics analysis of experimental samples was undertaken. RNA samples were sent to Edinburgh Genomics for Illumina library preparation and Illumina RNA-sequencing. Upon reception of the sequencing data, Illumina adaptors were trimmed from the raw read using the freely available software Cutadapt (Martin, 2011) and the quality of the trimmed reads was checked using the freely available software Fastqc (Andrews, *et al.* 2015). Reads under a quality Phred score of 30 were removed. The data was then uploaded to the publicly available Galaxy Web-platform (Afgan *et al.*, 2018) and a *de novo* assembly was constructed using the software Trinity (Grabherr *et al.*, 2011). Contigs less than 300bp were removed from the *de novo* assembly and reads were thereafter aligned to the assembly using the Bowtie (Langmead *et al.*, 2009) and RSEM (Li and Dewey, 2011) methods. A raw count matrix was then constructed which was used in a differential expression analysis with the R package edgeR (Robinson, *et al.* 2010; McCarthy, *et al.* 2012). In the differential expression analysis, differentially expressed genes were defined as genes with a log fold change (logFC) either lower than -2 or higher than 2. A BLASTx search (Altschuh, *et al.* 1990) was performed for all differentially expressed genes against the NCBI *nr* protein database, using a e-value cut-off of 1e-5. Gene Ontology terms (GO) was retrieved from the UniProt database for differentially expressed genes with BLAST hits (Bateman, *et al.* 2017). A GO enrichment analysis using a Fisher exact test was finally performed on sponge genes using the R package TopGO (Alexa and Rahnenfuhrer, 2016). A summary workflow of the transcriptomic data analysis is provided in figure 3-3.

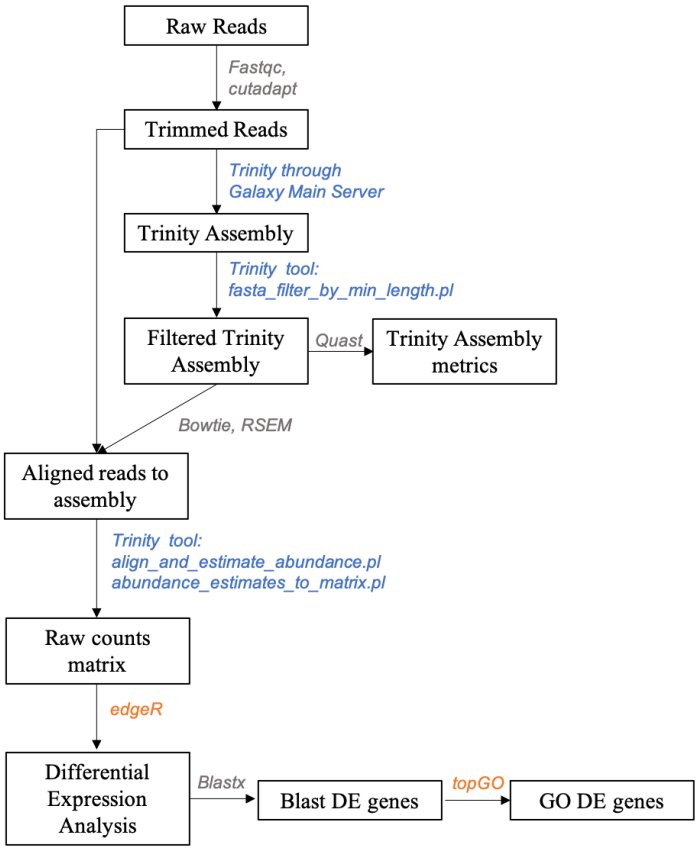

Figure 3-3. Summary workflow of the transcriptomic data analysis. Freely available programs are highlighted in grey. Steps involving the software Trinity are highlighted in blue. R packages are highlighted in orange.

Gene expression

Based on the transciptomics information gathered with the single concentration experiment, a series of qPCR primers were developed and used on the dose-response samples (Table 3-1). Quantitative PCRs (qPCR) of cDNA samples were undertaken using Primer Design (UK) Precision PLUS® master mix following manufacturer's instructions. Each reaction contained 10 µL of Precision PLUS® master mix, 0.6 µL 300 nM of forward primer, 0.6 µL 300 nM of reverse primer, 5 µL of cDNA template and 3 µL of water. A 48-well plate Applied Biosystems Step One thermocycler (ThermoFisher Scientific, UK) was used and conditions were set as follows: initial denaturation of 95.0° C for 10 min, 40 cycles of 95° C for 15 s, 15 s at primer melting temperature, and 95° C for 15 s, one minute at primer melting temperature and 15 s at 95° C.

Table 3-1. Primer sets characteristics. Primer name, sequence, position in the contig, annealing temperature (Tm) and efficiency (Eff.) is given for cyclophilin (Cyclo), cytochrome b5 (Cytb5), heat shock protein 70 (Hsp70) and tyrosine kinase (TK).

Primer	Primer sequence	Position	Tm	Eff.
Cyclo-F	5'-GCGAAGTCGTCGAAGGAATG-3'	445-464	58.5°C	0.973
Cyclo-R	5'-CCTCGATGATGCACCGTTGT-3'	534-515		
Cytb5-F	5'-CCAAGAGATTGCTGGTGGGT-3'	319-338	58.5°C	1.056
Cytb5-R	5'-GGTCATCTGGAGCTCGCATT-3'	531-512		
Hsp70-F	5'-AATTTCTCGTGAGCGGCCTT-3'	274-293	58.5°C	0.9623
Hsp70-R	5'-TCACCTCAGCGAGCAACAAA-3'	381-362		
TK-F	5'-CTCCCAGCTTGCCAAAGAGA-3'	349-368	58.5°C	1.093
TK-R	5'-TGCTAGCACTGGGATTGTGG-3'	635-616		

For every primer set used, amplification was first tested by PCR on an Applied Biosystems Veriti 96 well thermocycler (ThermoFisher Scientific, UK) using Qiagen (UK) *Taq* PCR master mix following manufacturer's instructions. Each reaction contained: 12.5 µL master mix, 0.5 µL forward primer, 0.5 µL reverse primer, 1 µL cDNA template, 0.5 µL dimethyl sulfoxide and 10 µL water. Thermocycler conditions were set to: initial denaturation of 95.0° C for 2 min followed by 30 cycles of 95° C for 30 s, 60 s at primer melting temperature, 72° C for 1 min, and then 72° C for 5 min. PCR results were checked by agarose gel electrophoresis, stained in GelRed™ (VWR, UK).

Optimisation was then performed to determine the best qPCR conditions to be applied for each primer. All primer sets used in here have efficiencies between 90-100%. Relative fold change in expression of each target gene was then determined using the $\Delta\Delta C_t$ method (Henry *et al.,* 2009).

3.3.6. Histological investigation

Histological examination of tissue samples under light microscopy was undertaken on the high dose-response samples to determine the cellular impact of crude oil and dispersed crude oil in *H. panicea*. The histological protocol described here was developed in collaboration with undergraduate student Fraser Dunnett (Heriot-Watt University) and based on the scientific literature available on sponge histology (Hoffmann *et al.*, 2003; Eerkes-Medrano *et al.*, 2015). Small tissue samples (less than $0.5cm^3$) were preserved in 10% neutral buffered formalin solution. Samples were then dehydrated in an ethanol series (70%, 90% and 100% EtOH), cleared in xylene and

embedded in paraffin. Paraffin sections (8µm) were then obtained using a Historange LKB Bromma 2218 microtome. Sections were stained with haematoxylin and eosin and slides were observed on a Zeiss Axio Scope.A1 microscope under brightfield. Images were captured using an AxioCam ERc 5s (Carl Zeiss, Germany).

Both positive (with pronounced tissue lesions) and negative controls (with healthy tissue) were needed to allow for comparison with experimental samples. Negative controls were collected from the field and directly preserved for histological investigation. Positive controls were also prepared by injecting freshly collected *H. panicea* samples with crude oil with a glass syringe and left in aquaria for 48h before being preserved. Furthermore, identification of specific tissue structures or cell types were conducted with the Thesaurus of Sponge Morphology (Boury-Esnault and Rützler, 1997).

3.4. Results

3.4.1. Analytical chemistry

Gas chromatography-mass spectrometry analysis of water samples was performed to determine the concentration of 16 PAHs. Overall individual hydrocarbon concentration measured varied from 0 to 13452.4 µg/L. Phenanthrene and fluorene were the two most prominent hydrocarbons measured in our analysis (Figure 3-4). Total concentration (sum of all hydrocarbons analysed) varied between 0.02 µg/L (WAF 0.5 g/L of crude oil) and 35758.0 µg/L (CEWAF 10.0 g/L of crude oil). For each hydrocarbon analysed, concentration measured overall increased in WAF and in CEWAF samples with increasing oil loading. For the same oil loading, the concentration of hydrocarbons in the CEWAF samples were, overall, higher than concentration determined in the WAF samples. Furthermore, complex 5 or 6 ring aromatics (from benzo[A]fluoranthene to indeno[1,2,3,C,D]pyrene) were predominantly found in CEWAF samples at high oil loading (Figure 3-4). Dibenz[A,H]anthracene and benzo[G,H,I]perylene were not detected in any samples (Figure 3-4).

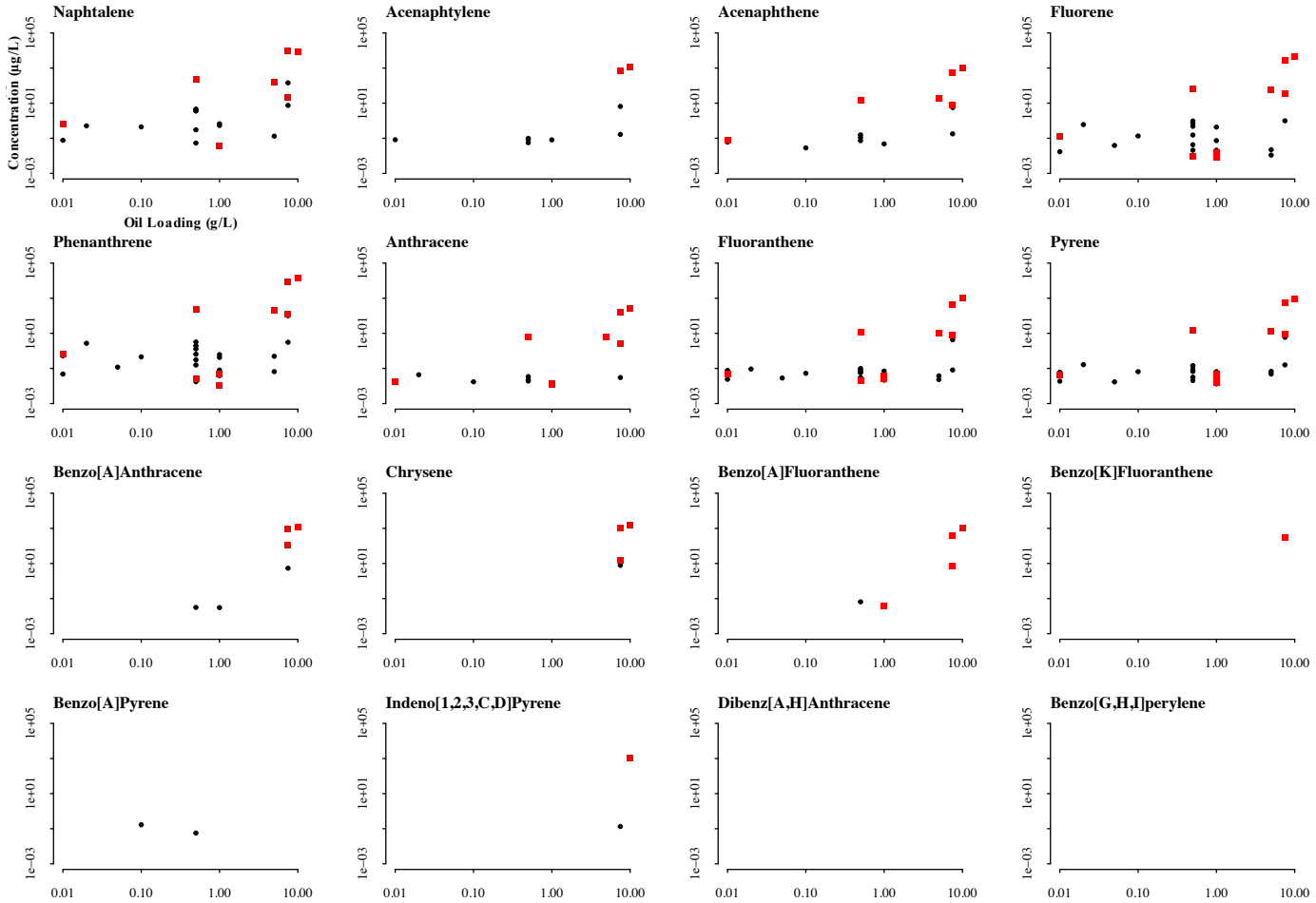

Figure 3-4. Hydrocarbon concentrations across oil loadings. In each plot, black dots are for WAF samples and red squares are for CEWAF samples.

3.4.2. Gross observations

All *H. panicea* samples kept in control conditions survived the experiments, appeared healthy and still displayed their natural sharp yellowish green colours at the end of the experiments. Furthermore all *H. panicea* samples survived the 48 h exposure to DMSO, BaP and WAF during the single concentration experiment and the 48 h exposure to WAF in the dose response experiments (at all oil loadings). These samples also appeared healthy as no signs of external tissue damage could be detected, and their colour remained unchanged until the end of the experiments. When considering the CEWAF treatment, all sponges survived the exposure in the single concentration experiment (1g/L of oil with dispersant). However, some samples exposed to the highest oil loading in the CEWAF dose response experiment showed significant rapid external tissue decay, displaying patches of dark tissue and died within the first 24 h of the total 48 h exposure. Specifically, sponges exposed to 3.5 g/L, 7.5 g/L (one of the two replicates survived) and 10 g/L of oil with dispersant did not survive the exposure. Physiological, histological and molecular data for these samples are therefore not available.

3.4.3. Physiology

Respiration rates between individuals across the single concentration experiment varied greatly. Overall, respiration rate ranged from 0.6 (BaP ind. 1 after exposure) to 35.3 µmol cm^{-3} $hour^{-1}$ (Control ind. 3 during exposure) (Figure 3-5). No clear pattern could be determined (Figure 3-5) and no statistical differences between treatment (*p*-value=0.34), time (*p*-value =0.53) or time*treatment (*p*-value =0.89) were detected by the repeated-measures ANOVA (Table 3-2). Clearance rates also varied between individuals and throughout the single concentration experiment, but a clear pattern could be detected in these data (Figure 3-5). A sharp decrease in clearance rate was measured in all samples exposed to WAF, CEWAF and BaP during the exposure and the clearance rate remained low 48 h after the end of the exposure (Figure 3-5). All values measured in samples in control and DMSO conditions across the experiment as well as in samples before exposure in hydrocarbon treatments ranged between 0.5 (Control ind. 3 before exposure) and 4.9 cm^3 cm^{-3} min^{-1} (WAF ind. 3 before exposure). In the hydrocarbon treatment conditions (WAF, CEWAF and BaP) during and after exposure, clearance rate decreased to a minimum of 0.1 cm^3 cm^{-3} min^{-1} (WAF ind. 1 after exposure). Time (*p*-value=1.61e-08) and Treatment*Time (*p*-value=3.01e-05) appeared as strongly statistically significant in the repeated-measures ANOVA (Table 3-2).

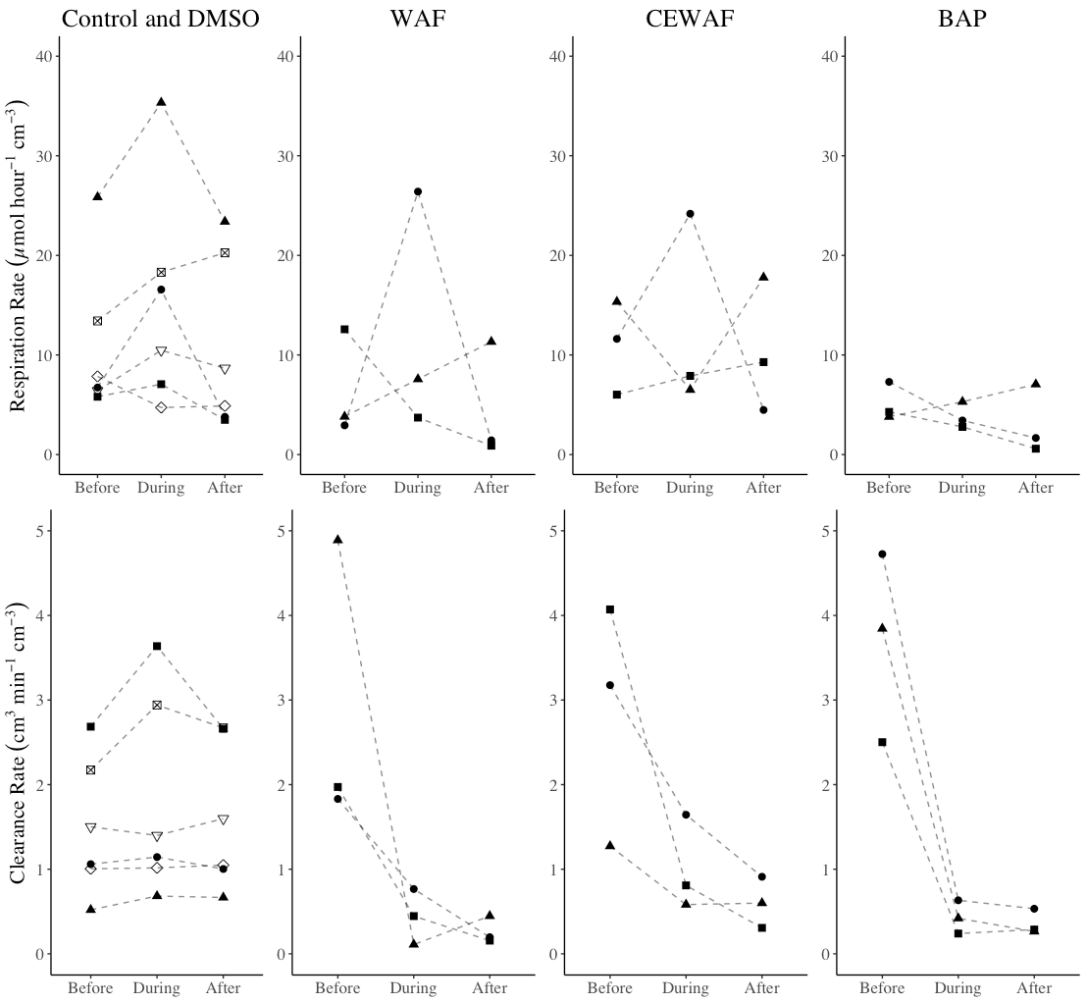

Figure 3-5. Single concentration exposure experiment physiology measurements. In the first panel, full symbols represent control sample measurements whereas empty symbols represent DMSO sample measurements.

Table 3-2. Results of the repeated-measures analysis of variance (ANOVA) on respiration and clearance rates from the single concentration exposure experiment. Element in bold highlight statistical differences

Response Variable	Explanatory variable	χ^2	Degrees of Freedom	Pr(>χ^2)
Respiration rate	Treatment	4.4808	4	0.3448
	Time	0.3949	1	0.5298
	Treatment*Time	1.1318	4	0.8892
Clearance rate	Treatment	0.8513	4	0.9314
	Time	**31.9094**	**1**	**1.615e-08**
	Treatment*Time	**26.1080**	**4**	**3.010e-05**

Physiological data gathered in the dose-response exposure experiments were in accordance with the data collected during the single concentration experiment. Respiration rate varied across individuals in both the WAF and CEWAF experiments and ranged from 1.1 (7.5 g of oil/L with dispersant) to 22.9 µmol cm^{-3} $hour^{-1}$ (1 g of oil/L) (Figure 3-6). No clear pattern was detected in the data (Figure 3-6) and no dose-response models appeared to fit the data or to detect any effect of oil loading on respiration rate. Clearance rate rapidly decreased with increasing oil loading in both the WAF and CEWAF dose-response experiments (Figure 3-6).

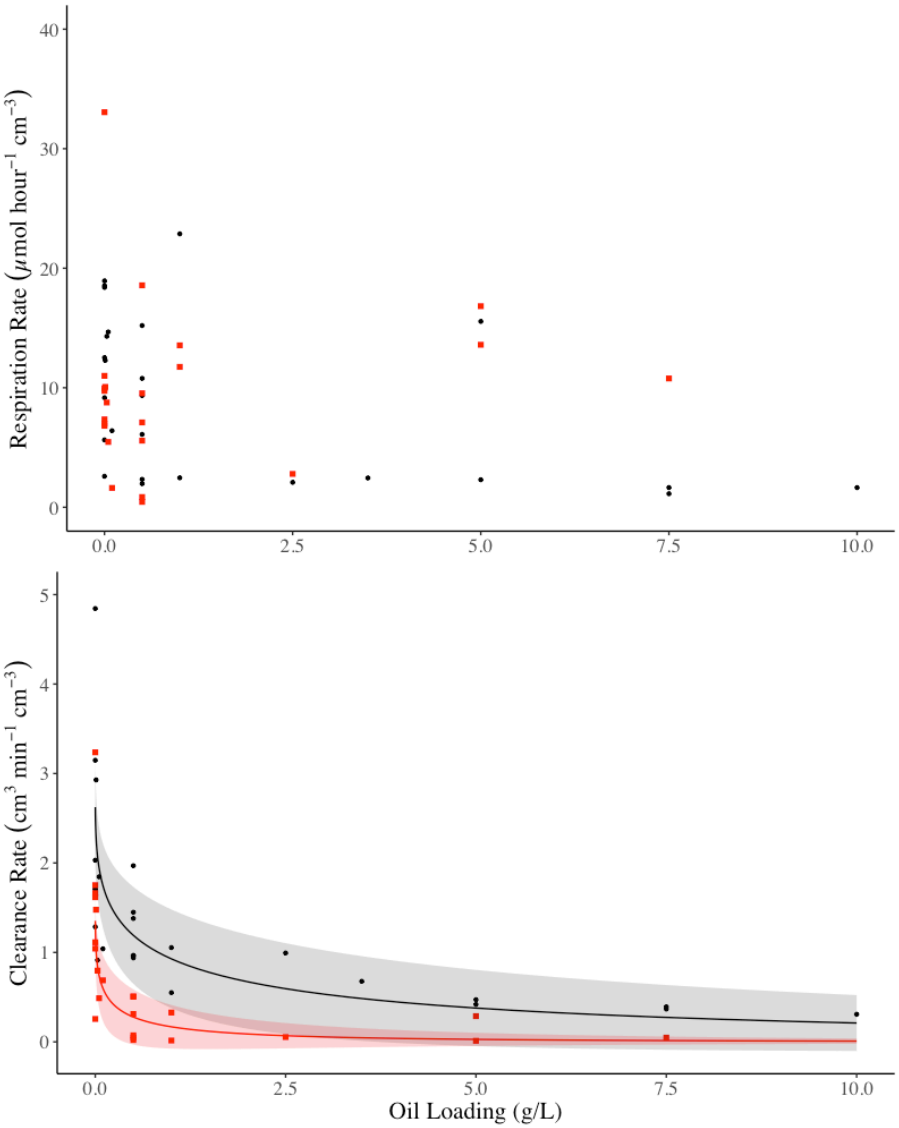

Figure 3-6. Dose-response physiology measurements. WAF measurement are in black dots and CEWAF measurements are in red squares. The clearance rate dose-response model is plotted in black for WAF samples and in red for CEWAF samples. Ribbons around each curve represent the confidence intervals.

Clearance rate in control conditions varied from 0.3 to 5.5 cm^3 cm^{-3} min^{-1} across WAF and CEWAF experiments. The lowest clearance rate in the WAF exposure only reached 0.3 cm^3 cm^{-3} min^{-1} (at 10 g of oil/L) while the lowest clearance rate measured in the CEWAF experiment dropped to 0.01 cm^3 cm^{-3} min^{-1} (at 5 g of oil/L with dispersant). Overall clearance rates in samples exposed to CEWAF were lower than those measured in samples exposed to WAF at the same oil loading. All coefficients of the Weibull 1 log-logistic dose response model (slope, upper asymptotic limit and ED50) were statistically significant (Table 3-3). The model fitted the data well (lack of fit test *p*-value=0.84) and a dose effect on clearance rate was statistically significant (*p*-value=6.4e-07) (Table 3-3). CEWAF ED50 (0.04 g/L of crude oil with dispersant) differed statistically from WAF ED50 (1.56 g/L of crude oil), however the slopes for each dataset did not differ significantly (Tables 3-3 and 3-4).

Table 3-3. Results of the dose-response model applied to clearance rate from the dose-response experiments. Elements in bold highlight statistical differences

Parameters estimates				
	Estimate	Std Error	t-value	*p*-value
Slope WAF	**0.369172**	**0.120051**	**3.0751**	**0.000104**
ED50 WAF	**1.560996**	**0.482497**	**3.2458**	**0.003609**
Slope CEWAF	**0.326211**	**0.076470**	**4.2659**	**<2.2e-16**
ED50 CEWAF	**0.043311**	**0.018123**	**2.3910**	**0.021145**
Upper Asymptote	**2.229147**	**0.070923**	**31.4304**	**0.002242**
Lack Of Fit Test				
Model Degrees of Freedom	RSS	Degrees of Freedom	F-value	*p*-value
44	31.901	17	0.6226	0.8446
No Effect Test				
	χ^2 test	Degrees of Freedom	*p*-value	
	3.431826e+01	**4**	**6.411789e-07**	

Table 3-4. Results of parameter comparison tests between dose-response model estimates. Elements in bold highlight statistical differences.

Parameter compared	Estimate	Std Error	t-value	*p*-value
ED50	**0.027668**	**0.045363**	**-21.434**	**<2.2e-16**
Slope	0.88363	0.87114	-0.3458	0.7311

3.4.4. Transcriptomic analysis

Transcriptomic analysis of samples collected after exposure in the single concentration experiment was performed. Total number of reads obtained from the Illumina sequencing for each treatment before and after trimming and quality check are given in table 3-5. The quality of reads was high across samples and treatments and over 236 million reads were used for the *de novo* assembly (Table 3-5). The *de novo* assembly obtained through Trinity was constituted of 235 561 contigs, all above 300 bp (Table 3-6). 47 433 contigs were larger than 1000 bp. The assembly had a GC content of 45.54% and a N50 of 984 (Table 3-6).

Table 3-5. Transcriptomics data summary. Total number of reads (N reads) before (BT) and after (AT) trimming, GC content and sequence length after trimming (AT) is provided.

Condition	N reads BT	N reads AT	GC content (%)	Sequence length AT
Control	51 211 739	41 009 710	47	75
DMSO	58 776 288	47 128 808	48	75
WAF	61 647 651	49 201 885	48	75
CEWAF	56 181 414	44 729 898	48	75
BaP	68 140 135	54 859 604	48	75

Table 3-6. Statistics of the Trinity *de novo* assembly. Total number of contigs, GC content (GC) and standard statistics (N50, N75, L50, L75) are provided.

Filtered Assembly Statistics	
Total number of contigs	235 561
GC (%)	45.54
N50	984
N75	512
L50	48 509
L75	115 109

In total, 1917 genes were found to be statistically differentially expressed across all treatments compared to control samples (Figure 3-7). The largest number of differentially expressed genes (1461) was detected in the CEWAF treatment while the

smallest number of differentially expressed genes (237) was found in the DMSO treatment (Figure 3-7).

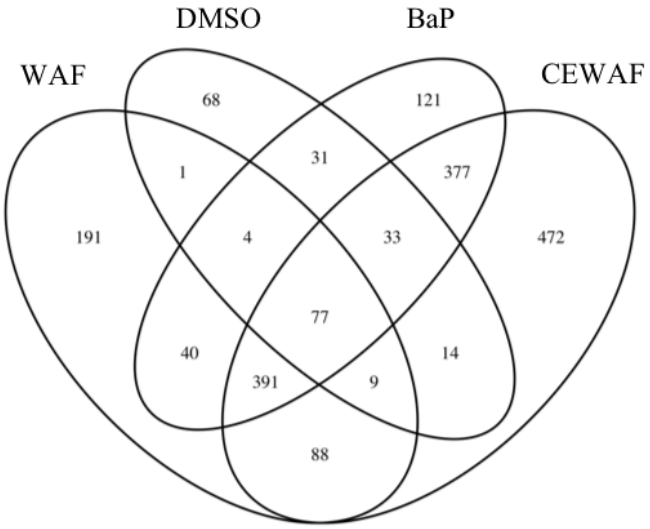

Figure 3-7. Venn diagram of the number of differentially expressed genes in each treatment compared to control.

Figure 3-8 shows the ordinated log fold change (log FC) of the 1461 differentially expressed genes from the CEWAF treatment compared to their respective log FC in the WAF (Figure 3-8A), BaP (Figure 3-8B) and DMSO (Figure 3-8C) treatments. Overall, genes up/down regulated in the CEWAF treatment were also up/down regulated in the WAF and BaP treatments. Fold change direction (negative or positive) and scale appeared consistent for a majority of genes when considering hydrocarbon treatments (Figure 3-8AB). This is in sharp contradiction with what can be observed in the DMSO treatment where most differentially expressed genes identified in the CEWAF treatment appear not to be differentially expressed in the DMSO treatment (Figure 3-8C).

Differentially expressed genes were blasted against the NCBI *nr* database. Blast of the 1917 differentially expressed genes in our experiment returned hits for 32% of the contigs. Of the 631 genes with hits, 54.5% originated from Metazoan and Porifera sequences (Figure 3-9). Eukaryota sequences accounted for 30.8% of the hits retrieved while bacteria, archaea and viruses respectively made up 13%, 0.6% and 1.1% of the blast hits (Figure 3-9). Amongst the host genes, genes involved in the regulation of oxidative stress, in the control of cell cycling, in stress response and MAPK pathway were identified (Table 3-7).

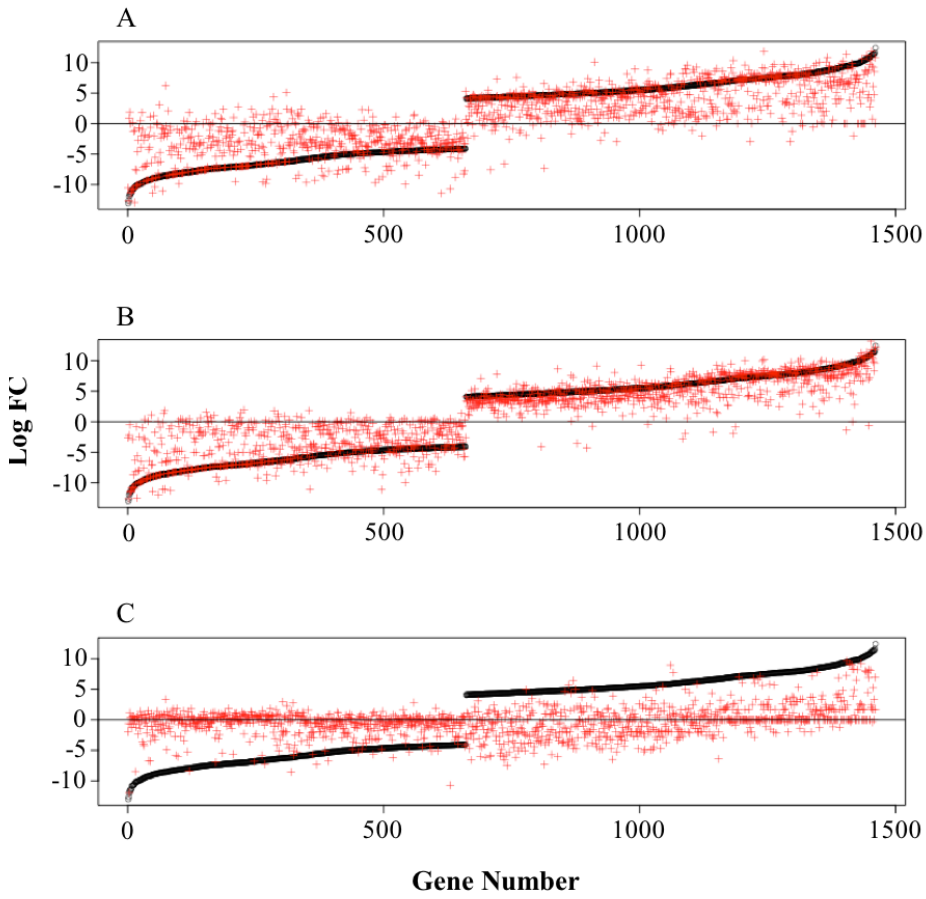

Figure 3-8. Scatterplot of the ordered ordinated differentially expressed genes in the chemically enhanced water-accommodated oil fraction treatment (black dots) compared to the remaining treatments (red crosses). (A) WAF. (B) BaP. (C) DMSO.

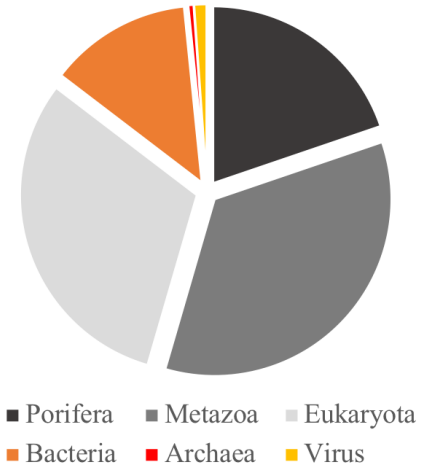

Figure 3-9. Origin of blast hits for differentially expressed genes with successful blast identification.

A total of 63 up-regulated genes, from Porifera, Metazoa and Eukaryota hits, were retrieved for the GO analysis terms (Figure 3-10). Eukaryota genes were included in this analysis as *H. panicea* harbours extremely few eukaryotic symbionts. Thirty-one

GO terms were significantly enriched within the host genes. Genes playing a role in cell adhesion, one carbon metabolic processes, macromolecule metabolic processes as well as defence response were significantly enriched (Figure 3-10). Further analysis of the expression levels of these genes (25 in total) was performed through the production of a heatmap (Figure 3-11). The heatmap revealed that hydrocarbons samples were characterised by high expression levels for all of these genes, confirming the findings of the GO analysis.

Table 3-7. Gene families amongst sponge differentially expressed genes relevant to hydrocarbon detoxification and cellular stress response.

Gene Family	Number of contigs identified
Cyclophilin	1
Cytochrome b	3
Cytochrome c	1
Flavin-containing monooxygenase	1
Heat shock proteins	9
Inhibitor of Apoptosis Proteins	1
MAP3K7 binding protein 1	1
TNF receptor-associated factors	11
Universal stress proteins	2

3.4.5. Gene expression

To confirm the findings of the transcriptomic study in the single concentration experiment, qPCR on the dose-response samples was performed using one reference gene (tyrosine kinase) and three target genes (cyclophilin, cytochrome b5, Hsp70). qPCRs for all four genes were undertaken on cDNA originating from the WAF and CEWAF dose-response samples. All three target genes were up-regulated in WAF and CEWAF samples from the lowest to the highest oil loading. High variation in log FC between samples was, however, observed. For cyclophilin, log FC in exposed samples varied between 1.27 and 24.7. For cytochrome b5, log FC in exposed samples varied between 1.29 and 16.6. For Hsp70, log FC in exposed samples varied between 1.02 and 13.0. No dose response relationship between the log FC and the oil loading was found for any of the genes

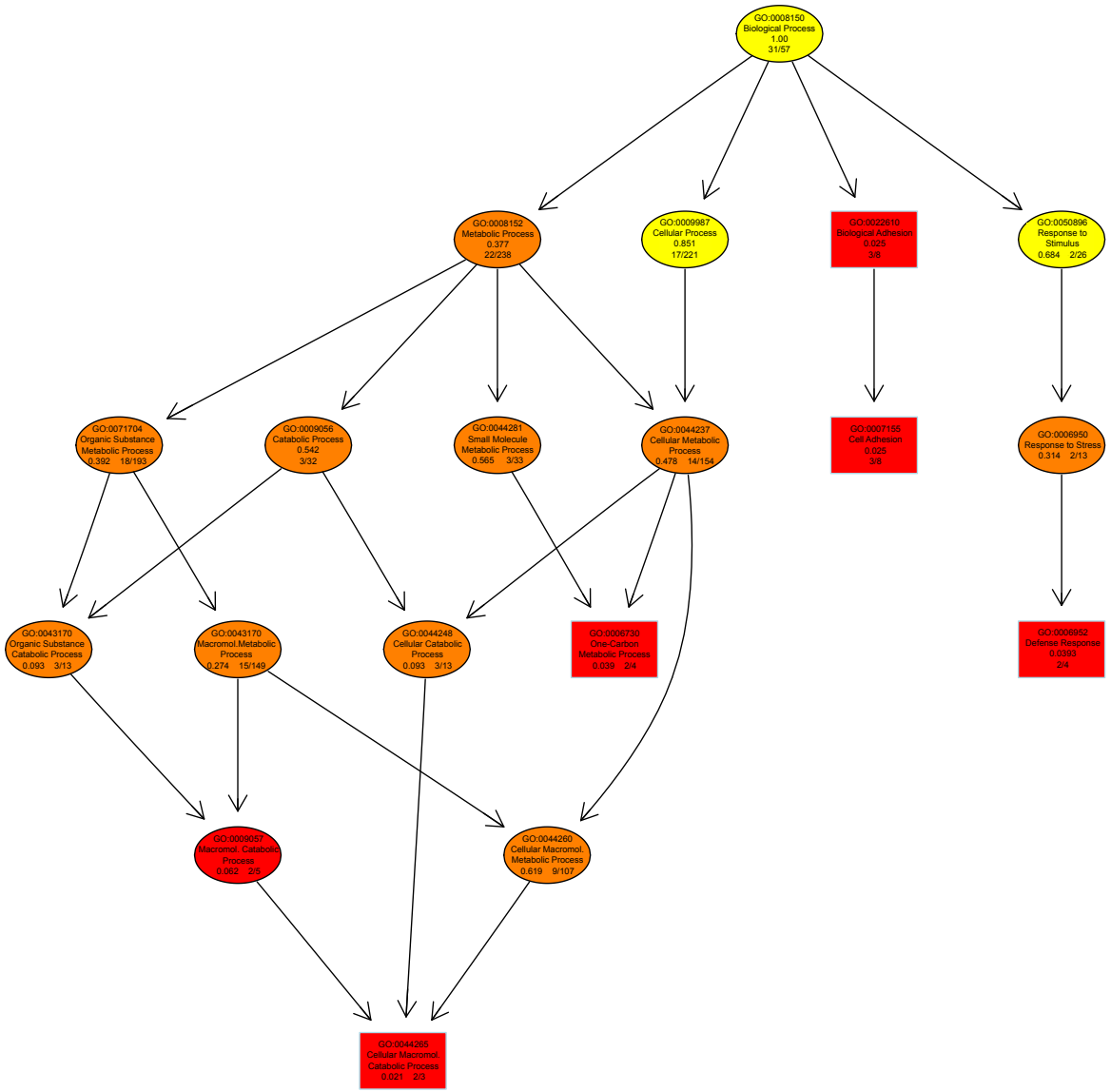

Figure 3-10. Results of the gene ontology (GO) enrichment analysis performed on sponge up-regulated genes. In each compartment, GO identification numbers and names are given, followed by the *p*-value and finally, the ratio of the number of genes detected to the number of genes expected at random. Square compartment highlights significantly enriched GO terms. The colours of the compartment highlight the level of significance (yellow, not significant, red highly significant).

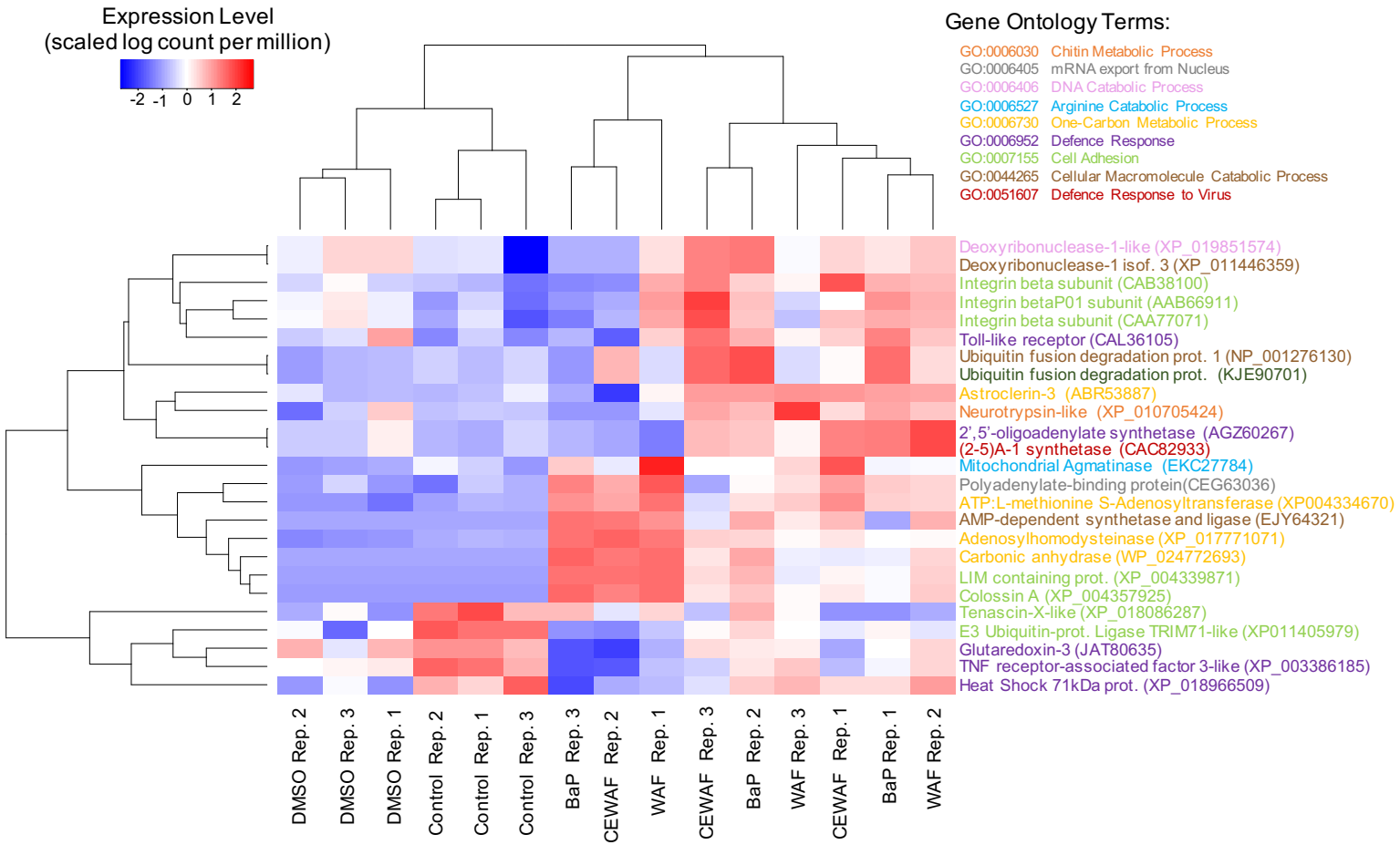

Figure 3-11 Heatmap of the level of expression of all enriched genes detected in the gene ontology (GO) analysis. Gene ontologies are colour coded for clarity.

3.4.6. Histology

To understand the impact of high oil loading WAF and CEWAF on *H. panicea* tissue, a histological investigation of a subset of the dose response experiments was conducted. Negative control samples collected from the field and preserved right away as well as control samples from the dose-response experiments displayed similar tissue organisation. Chambers and channels through which water were pumped, could clearly be seen in the sections, surrounded by dense sponge tissue (Figure 3-12A).

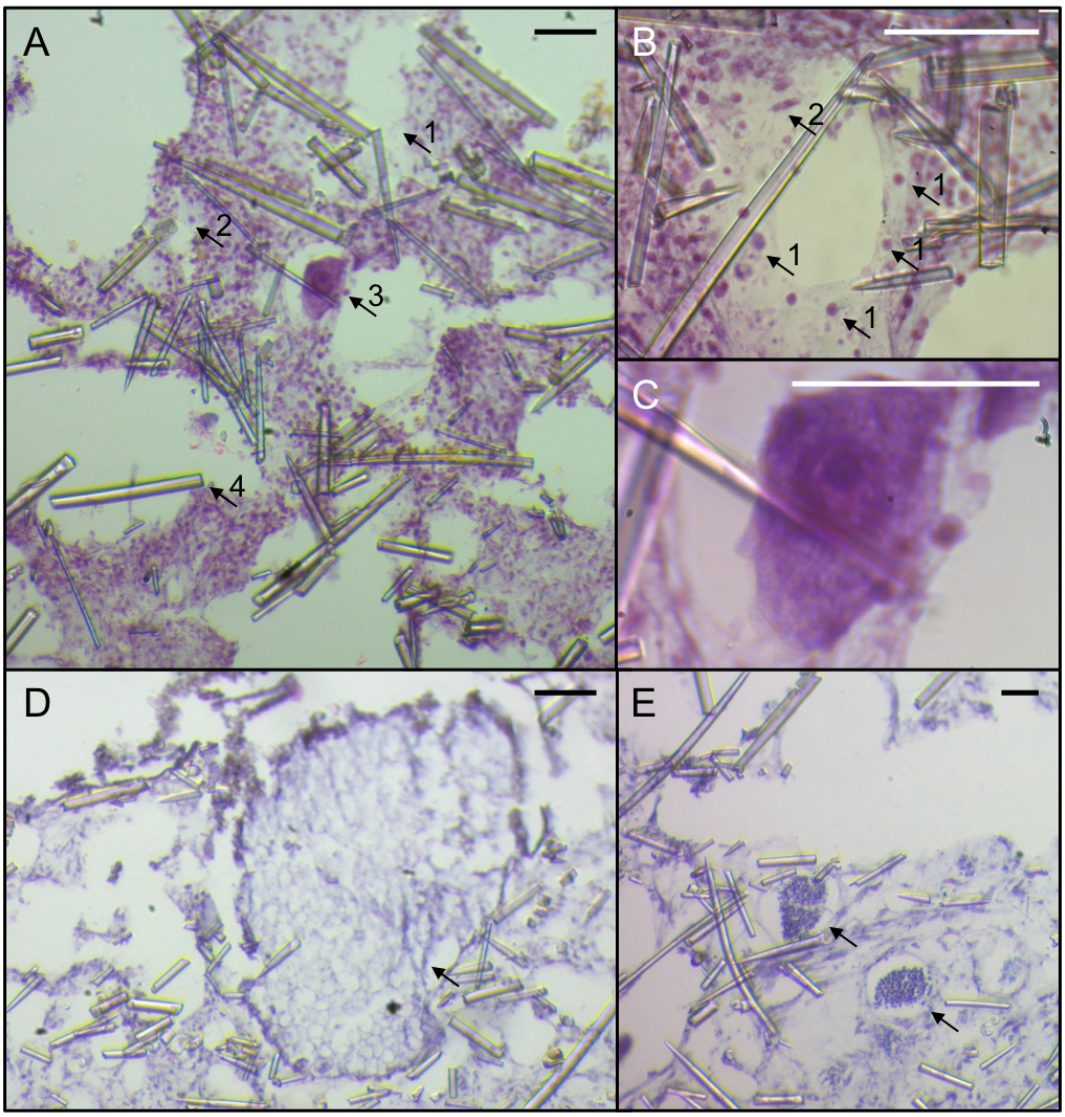

Figure 3-12 Histological investigation of sponge tissue. (A) Healthy sponge tissue with spongin (1), choanocytes (2), archeocytes (3) and spicule fragments (4). (B) Choanocyte chamber in healthy sponge tissue with (1) choanocytes and (2) actinocyte. (C) Archeocyte. (D) Area of cell decay in hydrocarbon exposed samples. (E) Accumulation of cell debris in choanocyte chambers in hydrocarbon exposed samples. The scale bar represents 50µm.

Spongin, a skeletal substance made of collagen, was visible in some areas of the tissue (Figure 3-12A). Choanocytes, the sponge pumping cells, could be seen lining some of the

channel walls (Figure 3-12AB). Actinocytes, smaller elongated cells, were less frequently observed but could sometimes be detected next to the choanocytes (Figure 3-12B). Large archeocytes, cells capable of phagocytosis, were present in numbers in the tissue and could be recognised by their large nuclei and denser cytoplasmic contents (Figure 3-12AC). Spicules and spicules fragments were spread out in the sections, with no specific organisation probably due to the microtome sectioning step of the histological methodology (Figure 3-12ABC). Positive control samples (injected with crude oil), (10.0 g/L of oil loading) and CEWAF (7.5 g/L of oil loading with dispersant) samples displayed, overall, a similar tissue organisation to the controls, but the tissue seemed overall less dense. Furthermore, accumulation of material within the tissue (Figure 3-12D) and in lumina (Figure 3-12E) which was not detected in the control samples was also observed within the tissue of positive control, WAF and CEWAF exposed samples.

3.5. Discussion

3.5.1. Physiological impacts of crude oil and dispersed crude oil contaminated seawater on the sponge *Halichondria panicea*

Both respiration rate and clearance rate were measured during the exposure experiments conducted in this study but only clearance rate was impacted by exposure to hydrocarbons. Respiration rates from the single concentration experiment and the dose-response experiments were highly variable and did not seem to change with exposure to hydrocarbons. The high variability seen in my measurements is, however, in accordance with the literature available for *H. panicea* (reviewed in Osinga *et al.,* 1999). Clearance rates measured in both the single concentration experiment and in the dose-response experiments also displayed high inter-individual variability, which is in accordance with literature available for other encrusting sponges (Goeij *et al.*, 2008a). However, in contrast to respiration rate, clearance rate decreased sharply in sponges exposed to WAF or CEWAF, even at low oil loading. A decrease in filtration rate was also observed in *H. panicea* exposed to cadmium (Olesen and Weeks, 1994), showing that this appears to be a typical physiological response to pollutants in *H. panicea.* Concentration of cadmium over 100 µg/L led to longer term filtration loss with filtration rate remaining low for several hours after exposure (data only available for 4 h in the study) (Olesen and Weeks, 1994). This is also in accordance with observations made in the single exposure experiment where sponges exposed to hydrocarbon treatments (WAF, CEWAF and BaP) displayed lowered clearance rate even 48 h after the end of the exposure. It is likely that stopping its filtration activity for extensive periods of time will strongly impact survival

of *H. panicea*. The capacity of sponges to survive longer exposure periods should therefore be investigated.

3.5.2. Molecular impacts of crude oil and dispersed crude oil contaminated seawater in sponge *Halichondria panicea*

Large numbers of differentially expressed genes were detected in samples exposed to hydrocarbons (WAF, CEWAF and BaP treatments) in my study. Furthermore, the expression profile of the differentially expressed genes was similar in samples exposed to hydrocarbons but not in samples exposed to DMSO. Exposure to hydrocarbons led to homogenous changes in gene expression in *H. panicea* holobiont. This confirms that BaP can be considered here as a positive control while DMSO can be regarded as a negative control alongside control (seawater) condition. This also suggests that genes involved in the detoxification of a single PAH alone, also play a role in the detoxification of a mixture of hydrocarbons (with or without dispersants). However, a strong increase in the number of differentially expressed genes was detected in the CEWAF treatment, highlighting the molecular impacts of dispersant in *H. panicea*. Results from the transcriptomics analysis was furthermore confirmed by qPCR with all three target genes being upregulated in all hydrocarbon treatments.

Amongst the sponge differentially expressed genes, genes involved in oxidative stress, general stress response and cell cycle regulation were differentially expressed. In many organisms, exposure to hydrocarbons can lead to a variety of biological response often mediated through the aryl hydrocarbon receptor pathway (Puga *et al.*, 2002). The aryl hydrocarbon receptor pathway has for example, been found to be upregulated in marine filter feeders exposed to hydrocarbons in the Gulf of Mexico during the Deepwater Horizon oil spill (Jenny *et al.*, 2016). The aryl hydrocarbon receptor is involved in oxidative stress response, cell cycle regulation and apoptosis (Nebert *et al.*, 2000); which is similar to the response seen in our study. Furthermore, the MAPK signalling pathway was shown to be activated in the sponge *Suberites domuncula* exposed to diesel oil WAF (Châtel *et al.*, 2011). In this chapter, genes implicated in the control of apoptosis and involved in the MAPK pathway were also detected. No clear single signalling pathway could, however, be identified from the Blast hits obtained in my study. Furthermore, most contigs from the de novo sequencing did not yield any blast hits. This could be due to the low number of Porifera genes available in the NCBI database (Riesgo *et al.*, 2012). Even if no specific signalling and detoxification pathways were singled out,

the transcriptomic analysis of my data does show that genes involved in stress responses in *H. panicea* are being expressed after exposure to hydrocarbons.

The gene ontology enrichment analysis offered further insights into the role of the up-regulated genes in the sponge samples. Genes involved with the defence response including the defence response against viruses were significantly enriched in the treatment samples compared to controls and were highly expressed in the hydrocarbon samples. This shows that the immune system of the sponge is impacted by the hydrocarbon contamination, which has also been found to be the case in experimental work on other filter feeding organisms (Boutet *et al.* 2004). Metabolic processes from small molecule metabolic process to macromolecule metabolic processes were also enriched in the sponge samples. Sponges exposed to hydrocarbons reduced strongly their filtration rate and hence their intake of nutrients and carbon. Changes to metabolic processes could indicate that the sponges are reacting to this lower carbon intake by modify their metabolism.

3.5.3. Implication for the use of dispersants in sponge-dense areas

Analytical chemistry conducted on water samples from the experiments conducted in our study revealed that hydrocarbons increased in concentration with oil loading. Concentrations found in my samples are lower than those described in the protocol followed to produce WAF and CEWAF solutions (Aurand and Coelho, 2005). This difference is due to the larger incubation chamber used in my study to allow experimentation on sponges. Despite being lower than the CROSERF protocol, concentrations measured in my samples at the lower oil loadings are comparable to what was measured in surface seawater during the Deepwater Horizon oil spill (Diercks *et al.*, 2010).

At a given oil loading, the concentration of hydrocarbons was higher in CEWAF than in WAF samples. CEWAF samples were also characterised by the presence of more complex PAHs. This could explain the lower clearance rate (ED50) measured in sponges exposed to CEWAF compared to WAF, as well as the fact that CEWAF samples displayed the highest number of differentially expressed genes. It seems here, that the use of dispersant has had a negative effect on *H. panicea* by increasing the bioavailability of PAHs. The use of dispersant as a spill-remediation technique in an area of high sponge density should therefore be carefully considered.

3.5.4. Histological impacts of crude oil and dispersed crude oil contaminated seawater in sponge *Halichondria panicea*

The histological investigation of samples exposed to high oil loading WAF and CEWAF conducted in this study showed that exposure to crude oil and dispersed crude oil contaminated seawater lead to significant tissue damage in *H. panicea*. The overall tissue organisation observed in our study is in accordance with previous *H. panicea* tissue description (Eerkes-Medrano *et al.*, 2015). In positive control samples as well as in experimental samples exposed to WAF and CEWAF, the tissue appeared less dense and presented tissue lesions not visible in control specimens. Diseased sponge tissue can also display histological lesions with incomplete choanocyte chambers and accumulation of high levels of debris in the remaining chambers (Luter *et al.*, 2010). Other studies have reported that marine sponge tissue can regress under stress conditions but it also has the capacity to recover back to a healthy state when conditions improve (Thoms and Schupp, 2008; Luter *et al.*, 2012). Regressed tissue displays fewer choanocyte chambers and densely packed granulated or spherulous cells (Thoms and Schupp, 2008; Luter *et al.*, 2012). No accumulation of granulated or spherulous cells was observed in my samples and it seems the damage caused by hydrocarbon exposure in *H. panicea* is more comparable to the damage described in diseased sponges.

3.6. Conclusion

This chapter demonstrated that clearance rate in *H. panicea* decreased sharply when exposed to WAF and CEWAF but respiration rate did not seem impacted by the contamination. Furthermore, transcriptomic analysis revealed a homogenous gene expression response across all hydrocarbon treatment tested (WAF, CEWAF and BaP). Transcriptomic analysis also showed that differentially expressed genes from both the sponge host as well as the sponge symbionts were impacted by the contaminated seawater. Genes involved in the defence response and response to viruses were specifically up-regulated in the sponge host. Finally, high concentrations of WAF and CEWAF also led to tissue lesions consistent with necrosis in *H. panicea.*

In the aftermath of an oil spill, sponges can not only be exposed to hydrocarbons through contaminated seawater but also through the resuspension of contaminated sediments. Chapter 4 will present the experimental work conducted to test the impacts of contaminated sediments on H. *panicea* and its associated microbial community.

Chapter 4 Impacts of Crude Oil, Dispersed Crude Oil and Dispersant Contaminated Sediments on Model Sponge *Halichondria panicea* and Its Associated Microbial Community

4.1. Overview

The purpose of this chapter is to present the experimental work conducted on *Halichondria panicea* with hydrocarbon-contaminated sediments. A manuscript based this chapter will soon be finalised and submitted to the scientific journal Marine Ecology Progress Series (see appendix A).

4.2. Introduction

Once released into the sea, petroleum hydrocarbons will undergo a series of transformations. In marine environment, petroleum compounds will naturally be weathered through evaporation, dissolution, emulsification, photooxidation, sedimentation and biological biodegradation (National Research Council, 2003; Tarr *et al.*, 2016). The relative importance of each of these processes will depend both on the properties of the petroleum released (density, viscosity, composition) and the physical and biological characteristics of the environment (temperature, mixing regime, depth of release) (National Research Council, 2003). The fate of the oil in the marine environment will also depend on the rate of release, usually much faster during an oil spill compared to a natural release at a seep (Shigenaka, 2011).

Sedimentation of hydrocarbons after an oil spill accounts for a significant part of the natural weathering process. After the Braer oil spill, offshore the Shetland islands, it is estimated that 35% of the oil spilled ended up in the sediments (Davies, *et al.* 1997). Once hydrocarbons reach the sediments, the microbial community associated with the sediment is altered (Kimes *et al.*, 2013; Bargiela *et al.*, 2015). Early studies showed that petroleum hydrocarbons can enhance (Bunch, 1987) reduce (Griffiths *et al.*, 1981) or have no effect (Bauer and Capone, 1985; Carman *et al.*, 1996) on total abundance of sedimentary bacteria. However, more recent studies have shown that abundance of oil-degrading bacteria, especially the ones capable of anaerobic hydrocarbons degradation, increases after exposure to hydrocarbons (Kimes *et al.*, 2013; Bargiela *et al.*, 2015). Degradation in sediment is usually slower than in seawater due to the oxygen and nutrient depleted conditions often characterising sediments (Liu *et al.*, 2012; Kimes *et al.*, 2013; Ziervogel *et al.*, 2016). Hydrocarbon associated with sediments, can therefore have a strong lasting influence on the local environment (Kingston, 2002; Liu *et al.*, 2012; Joye *et al.*, 2016; Ziervogel *et al.*, 2016).

Dispersants alters the behaviour of petroleum hydrocarbons in seawater. The use of dispersants in the remediation of an oil spill can lead to an increase in the concentration

of PAHs, which can cause sublethal and lethal effect in exposed marine organisms (Singer *et al.*, 1998; National Research Council, 2005). The formation of oil droplets in the water column after the application of dispersants is also of concern. Specifically how dispersed oil and oil droplets interact with sediments is still being investigated (Gong *et al.*, 2014; Sørensen *et al.*, 2014). Furthermore, dispersants have been shown to persist in marine sediments for up to 45 months after their use (White *et al.*, 2014).

The impact of contaminated sediments on ecologically relevant marine organisms such as sponges is unknown. During natural events of sediment resuspension, it is believed sponges can feed on particulate organic matter derived from the sediments (Hogg *et al.*, 2010). Active incorporation of sediment material such as quartz grains also contribute to other aspects of their biology, such as collagen production (Cerrano *et al.*, 2007). It is therefore also hypothesised that sponges can be exposed to contaminants from sediments in periods of sediment resuspension (Batista *et al.*, 2013). The biological impacts of crude oil and dispersed crude oil contaminated seawater on *Halichondria panicea* was determined in chapter 3. The impact of crude oil, dispersed crude oil and dispersant contaminated sediment, however, still needs to be studied.

The aim of this chapter is therefore to (1) produce crude oil, dispersed crude oil and dispersant contaminated sediment and monitor the impact on the associated microbial abundance, (2) determine the impacts of sediment contaminated with crude oil and/or dispersant mixtures on *H. panicea* physiology and gene expression and (3) determine the impact of oil contaminated sediment on the microbial community associated with *H. panicea*.

4.3. Material and methods

4.3.1. Sampling

For the purpose of this study, sponges, sediment and surface seawater samples were collected at Coldingham bay. Sponge samples were gathered as described in chapter 3: sponges were carefully removed with a scalpel from the rocks and placed into sampling bags filled with freshly collected seawater. Twelve sediment samples were collected using bespoke glass corers. Each glass corers of 50 mm in diameter and 100 mm in length were pushed approximately 40 mm into the soft sediment at low tide (Figure 4-1). The upper part of the tube was then tightly closed with a rubber bung. The tube could then be removed from the sediment without losing or disturbing the vertical distribution of the samples. A second rubber bung was used to close to second end of the tube in order to

safely transport the sediment samples (Figure 4-1). Samples were returned to Heriot-Watt University and kept in a cold-room at 7° C. Sponge samples were kept in a 35 L retention tank until the beginning of the experiment.

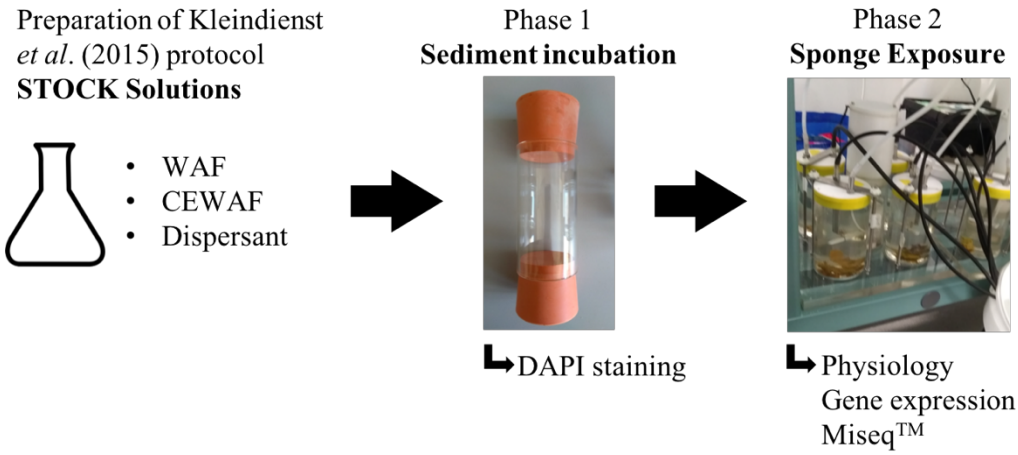

Figure 4-1. Workflow of experimental design. The experiment is be divided in two steps: phase 1 during which sediment samples in glass corers are exposed to control or treatment solutions and phase 2 during which sponges are exposed in incubation chambers are exposed to control or treated sediments.

4.3.2. Experimental design

The experiment design established in this chapter can be divided up into two phases: (1) sediment contamination to crude oil and/or dispersant mixtures and (2) sponge exposure to contaminated sediments (Figure 4-1). During the first phase, triplicates of sediment samples were exposed to water accommodated oil fraction (WAF), chemically enhanced WAF (CEWAF) and dispersant in seawater mixtures. In the control treatment, sediments were kept in uncontaminated seawater. During the second phase, sediments produced during the first phase were used to expose triplicate sponge samples to control sediments, WAF sediments, CEWAF sediments and dispersant-contaminated sediments, using the same incubation chambers described in chapter 3.

4.3.3. Preparation of contaminated sediments

Crude oil and dispersant used

As mentioned in chapter 3, Schiehallion crude oil (BP) and dispersant Slickgone NS (Dasic International) were used in all experiments presented in this thesis,. Schiehallon crude oil is produced at Schiehallion oil field in the Faroe-Shetland channel and was provided by BP. The crude oil is characterised by an American Petroleum Institute gravity of 25.2, a sulphur content of 0.46 % and a viscosity of 67 centistokes (cST) at 20°C (BP, 2017). Slickgone NS, provided by the Oil Spill Response Limited, is one of the dispersants approved for use by the United-Kingdom Marine Management

Organisation and is listed for potential use in the Faroe-Shetland channel in the case of a spill (BP, 2014; Marine Management Organisation, 2018).

The Kleindienst et al. *(2015) protocol*

The Kleindienst protocol is based on the methodology described in the supplementary data of Kleindienst *et al.* (2015). This protocol is based on the preparation of stock solutions of WAF and CEWAF that are then diluted into working solutions of WAF and CEWAF. For the preparation of one litre of WAF stock solution, 0.15 L of crude oil was mixed with 0.85 L of sterile seawater. 0.015 L of dispersants was added to the mixture for the preparation of CEWAF stock solution. The mixture is then mixed for 48 h. After mixing, the stock solutions are left to rest for one hour and 0.4 L of water fractions from the stock solutions are carefully removed from the mixture and added to 1.4 L of unfiltered seawater to obtain the WAF and CEWAF working solutions.

Several publications have called into question the protocol and conclusions explained in Kleindienst *et al.* (2015) (Lewis, 2015; Kleindienst *et al.*, 2016; Prince *et al.,* 2016). One of the objectives of the Kleindienst *et al.* protocol (2015) is to recreate conditions found in the water column during the Deepwater Horizon spill. The CROSERF report was set-up to provide consistent dispersant toxicity testing protocols. Considering both protocols in this thesis seem therefore adequate. Appendix C provide preliminary results of comparative experimental work conducted on *Myxilla sp* with both protocols.

Contaminated sediments

During the first experimental phase, sediment samples were kept in the collection glass corers, but the upper rubber rung was removed. A volume of 60.7 mL of collected seawater was added to each tube and gentle air mixing was provided for the next 48 h, to allow for the separate preparation of stock treatment solutions with the Kleindienst *et al.* (2015). Stock solution of WAF, CEWAF and dispersant in water were prepared with filtered sterile seawater following Kliendienst *et al.* (2015) and left air mixing for 48 h. Once the mixing period had ended, 17.3 mL of stock solution was added to each tube following the proportions of Kliendienst *et al.* (2015). For the control samples, 17.3 mL of filtered seawater mixed with seawater was added. All corers were then sealed with Parafilm® and kept for 48 h. Gentle air mixing was provided to the water above the sediments during the whole length of the experiment.

At the end of the first stage of the experiments, sediments samples were weighted (wet weight, WW) and subsamples were collected and frozen at -20° C. The remaining sediments (75 g WW per treatment) were then pull together within each treatment and used for the sponge exposure experiment. Sediments from each treatment were placed in 4 L conical flasks and 2.5 L of seawater was added into the flasks. Each conical flask was then be connected to the experimental set-up described in chapter 3. Rigorous air mixing was provided into each conical flask to allow partial mixing of the sediment and the water. Even so, some of the sediment did not appear to leave the conical flask and so the sponge samples were exposed ~15 g of sediments each. Sponges were kept in the incubation chambers for 48 h in clean seawater without sediments before the start of the exposure. After the acclimatisation phase, sponges were then exposed to sediments and seawater for 48 h.

4.3.4. Characterisation of contaminated seawater and sediments

406-diamidino-2 Phenylindole (DAPI) staining

At the end of the first phase of the experiment (sediment contamination), prokaryotic (bacteria and archaea) cell concentrations in sediments and in sweater was quantified by DAPI (406-diamidino-2 phenylindole) staining. DAPI staining was performed by PhD student Laura Duran Suja (Heriot-Watt University). Samples of water and sediments were fixed with 3.7% formaldehyde and stored at 4°C for a maximum of 2 weeks. For sediments, each sample was sieved with a 32um sieve using sterile seawater (passed through a 0.22um filter). For each fixed water sample, 5 ml was filtered (0.22 um) onto gridded (3 mm x 3 mm) polycarbonate filters – this volume was adjusted in order to achieve 10–150 cells per grid. The filters were mounted onto glass slides and the cells stained with DAPI (1 mg/ml) for 20 min and then counted under the Zeiss Axioscope epifluorescence microscope (Carl Zeiss, Germany). A minimum of 10 grids were randomly selected and photographed for cell counting. Concentration of bacteria was determined using the following formula:

$$N = \left(\frac{nb}{nSq}\right) Vf\left(\frac{A}{ASQ}\right)$$

where N is the total number of bacteria per mL, nb is the number of bacteria counted, nSq is the number of squares counted, Vf is the volume of seawater filtered, A is the effective filter area, and ASq is the area of one square of the grid.

Statistical Analysis of DAPI data

To test for statistical differences in DAPI cell counts, Shapiro and Bartlett tests were first conducted to verify the normal distribution and the homoscedasticity of the data. Both DAPI datasets were normally distributed. If variances across treatments were equal, analysis of variance (ANOVA) was then performed. If variances across treatments were unequal, one-way analysis of mean were conducted (Dalgaard, 2008). Pairwise t-test were performed finally with Holmberg adjusted p-values. All statistical tests were performed using the freely available software Rstudio (R core team, 2017).

4.3.5. Physiological endpoints

Sponge volume

The sponge volume was determined as explained in chapter 3. The volume of each sponge sample was determined at the end of each experiment. The thickness of the sample was measured directly with a calliper. A photography was then taken of each sample and the freely available software Fiji was used to determine the surface area of the sample (Schindelin *et al.,* 2012). Surface area was then multiplied by the thickness to determine the sponge volume.

Respiration rate

Respiration rate was determined as explained in chapter 3, at the end of the exposure. Respiration rate was determined by analysing the decrease in O_2 over time in each chamber. Concentration of O_2 in each chamber was measured every 15 seconds during two hours using Presens sensor spots connected to Oxy-4 optodes (Presens Precision Sensing GmbH, Germany). A two-hour time period was enough to detect a significant change in O_2 concentration in the respiration chamber. Stirring in the chambers was kept active during the measurement time but the inflow and outflow were closed so no fresh input of seawater was added. To account for microbial respiration in the seawater and sediments, blank measurement of respiration in seawater and treatment solutions were also determined in empty chambers. $\%O_2$ was converted to µmol/L using the R package presens (Birk, 2016). Sponge respiration rate were then determined using the following formula:

$$Respiration\ rate = (Resp_{Chamber} - Resp_{blank})/V_{sponge}$$

where $Resp_{chamber}$ is the respiration rate determined in the chamber with a sponge, $Resp_{blank}$ is the respiration rate determined in the blank chamber without a sponge and V_{sponge} the volume of sponge tissue (determined at the end of the experiment).

Clearance rate

Clearance rate was determined as explained in chapter 3, at the end of the exposure. *Isochrysis* Instant Algea® (Reed Mariculture, Californa) diluted solution was added to each chamber and the sponges were left to filter for two hours. Stirring in the chambers was kept active during the measurement time but the inflow and outflow were closed so no fresh input of seawater was added. Water samples were collected every 20 minutes and algae cell concentrations were determined through total absorbance measurements. Clearance rates for each sample was calculated as follows (De Goeij *et al.*, 2008a):

$$Clearance\ rate = (\left(\frac{V_{water}}{t}\right) \ln\left(\frac{C_0}{C_t}\right))/V_{sponge}$$

where V_{water} is the volume of water in the chambers, t the time of incubation, C_0 and C_t the initial and final concentration of algae in the chamber and V_{sponge} the volume of sponge tissue.

Statistical analysis of physiological data

To test for statistical differences in respiration and clearance rates after exposure to contaminated sediments, Shapiro and Bartlett tests were first conducted to verify the normal distribution and the homoscedasticity of the data. Both physiological datasets were normally distributed. If variances across treatments were equal, analysis of variance (ANOVA) was then performed. If variances across treatments were unequal, one-way analysis of mean were conducted (Dalgaard, 2008). Pairwise t-test were performed finally with Holmberg adjusted p-values. All statistical tests were performed using the freely available software Rstudio (R core team, 2017).

4.3.6. Sponge molecular endpoints

Tissue sampling

Tissue samples were collected at the end of the exposure to sediments. About 3 mm^3 of tissue was cut from individual sponge samples with a sterile scalpel and thoroughly rinsed with MilliQ™ water to remove any outer debris and seawater. Samples preserved in RNAlater® (Sigma-Aldrich, UK) and placed at -20° C.

RNA extraction

As explained in chapter 3, total RNA was extracted from sponge tissue samples using Qiagen (UK) RNAeasy® extraction kits following the manufacturer's instruction. Small sections of sample (about 1 mm^3) were homogenized using a MSE SoniPrep (UK) sonicator (5s burst at level 4) and lysed using the provide lysing buffer. DNA was

removed from the RNA extractions with Qiagen RNase-Free DNase sets. At the end of the protocol, RNA was eluded into 30 µL of DNA/RNA free sterile water. RNA quality and quantity were then assessed by spectrophotometer by NanoDrop™ (NanoDrop™ 2000, ThermoFisher Scientific (UK)). Only RNA samples with a 260/230 and 260/280 ratio of 1.8-2.2 were used in further analysis. RNA samples were stored in -80° C until sequencing or reverse transcription was performed.

Reverse transcriptase PCR (RT-PCR)

As explained in chapter 3, reverse transcriptase PCR (RT-PCR) was performed, to obtain cDNA, using nanoScript™ 2 (UK) Reverse Transcription kits following manufacturer's instructions. RNA samples were diluted to a concentration of 100 ng/µL prior to the RT-PCR. Each reaction contained 9.0 µL of RNA template, 1.0 µL of RT primers, 5.0 µL of nanoScript2 4X buffer, 1.0uL of dNTP mix, 3.0 µL of water and 1.0 µL of nanoScript2 enzyme. The annealing step of the RT-PCR was conducted at 65°C for 5min. After that, the extension step was carried out in 30 cycles of 42°C for 20min followed by 75°C for 10min. cDNA samples were stored at -20°C until qPCR was performed. Both RT-PCR steps were conducted on an Applied Biosystems Veriti 96 well thermocycler (ThermoFisher Scientific, UK).

Gene expression

As in chapter 3, quantitative PCRs (qPCR) were performed using primers targeting cyclophilin, cytochrome b5 (cyt b5) and hear shock protein (Hsp) 70. A gene coding for a tyrosine kinase was used a reference gene. qPCR of cDNA samples were undertaken, using Primer Design (UK) Precision PLUS® master mix following manufacturer's instructions. Each reaction contained 10 µL of Precision PLUS® master mix, 0.6 µL 300 nM of forward primer, 0.6 µL 300 nM of reverse primer, 5 µL of cDNA template and 3 µL of water. A 48-well plate Applied Biosystems Step One thermocycler (ThermoFisher Scientific, UK) was used and conditions were set as follows: initial denaturation of 95.0° C for 10 min, 40 cycles of 95° C for 15 s, 15 s at primer melting temperature, and 95° C for 15 s, one minute at primer melting temperature and 15 s at 95° C. For every primer set used, amplification was first tested by PCR on an Applied Biosystems Veriti 96 well thermocycler (ThermoFisher Scientific, UK) using Qiagen (UK) *Taq* PCR master mix following manufacturer's instructions. Each reaction contained: 12.5 µL master mix, 0.5 µL forward primer, 0.5 µL reverse primer, 1 µL cDNA template, 0.5 µL dimethyl sulfoxide and 10 µL water. Thermocycler conditions were set to: initial denaturation of 95.0° C for 2 min followed by 30 cycles of 95° C for

30 s, 60 s at primer melting temperature, 72° C for 1 min, and then 72° C for 5 min. PCR results were checked by agarose gel electrophoresis, stained in GelRed™ (VWR, UK). Relative fold change in expression of each target gene (log FC) was then determined using the $\Delta\Delta C_t$ method (Henry *et al.,* 2009).

Statistical analysis of gene expression data

To test for statistical differences in log FC of each target gene after exposure to contaminated sediments, Shapiro and Bartlett tests were first conducted to verify the normal distribution and the homoscedasticity of the data. All three datasets were normally distributed. If variances across treatments were equal, analysis of variance (ANOVA) was then performed. If variances across treatments were unequal, one-way analysis of mean were conducted (Dalgaard, 2008). Pairwise t-test were performed finally with Holmberg adjusted p-values. All statistical tests were performed using the freely available software Rstudio (R core team, 2017).

4.3.7. Characterisation of the sponge associated microbial community

DNA extraction

Total DNA extraction from tissue samples preserved in RNAlater (Sigma-Aldrich, UK) at -20°C were also performed. DNA was extracted using Qiagen (UK) DNeasy® Blood and Tissue kits following the manufacturer's instruction. Small sections of sample (about 1 mm^3) were homogenized using a sonicator (5s burst at level 4). At the end of the protocol, DNA was eluded into 30 µL of DNA/RNA free sterile water. DNA quality and quantity were then assessed by spectrophotometer by NanoDrop (NanoDrop™ 2000, ThermoFisher Scientific (UK)). Only DNA samples with a 260/230 and 260/280 ratio of 1.8-2.2 were used in further analysis. DNA samples were stored in -20°C until PCR was performed.

MiSeq™ sequencing

Barcoded 16S rRNA gene MiSeq™ sequencing, targeting the V4 hypervariable region, was employed to analyse the bacterial community associated with the *sponge H. panicea* (chapter 5). Firstly, amplification of 16S rRNA gene in 50uL duplicate reactions was undertaken. Each reaction comprised 32 µL of molecular biology grade water, 10 µL of 5x MyTaq™ polymerase reaction buffer, 2.5 µL of 4 uM primer mix, 0.5 µl of MyTaq™ enzyme (2.5U; BioLine), 3 µL of DMSO (6%), and 2 µL of gDNA. The primers used were 515f (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT) (Caporaso *et al.,* 2012). Both primers had Illumina

MiSeq™ overhangs attached to their 5′ ends. Both primers also had Illumina MiSeq™ overhangs attached to their 5' ends. Forward primers had golay barcodes added to the 5' ends. PCR conditions were set as follows: initial denaturation of 96°C for 1 min, 32 cycles of 96° C for 15 sec, 55° C for 15 sec, and 72° C for 30s, and a final extension at 72° C for 3 min. Secondly, samples were sequenced via the Illumina MiSeq™ platform (Illumina 2 x 250 V.2 kit) at Edinburgh Genomics (Edinburgh University, UK); sequences were demultiplexed prior to receipt at our laboratory. Subsequent processing of the Illumina sequence data was performed using the DADA2 package as wrapped in QIIME2 (assessed Aug 2018). In brief, paired end illumine reads were combined to form contiguous sequences. A fragment cut-off of 220 bp was established to maintain quality. These contigs were examined for low quality Phred scores and any identified chimeric sequences were removed. All quality approved sequences were compared on a single nucleotide resolution and the resulting single nucleotide variants were identified using the green genes database of 16S rRNA gene taxonomy. Composition of microbial diversity was examined at family level and alpha-diversity was assessed in the samples through rarefaction analysis on observed operational taxonomical units. Miseq™ data processing was performed in collaboration with PhD student Laura Duran (Heriot-Watt University, UK) and Steve Summers (Nanyan Technological University, Singapore).

4.4. Results

4.4.1. DAPI cell counts

Bacterial abundance in seawater and sediment was assessed using DAPI staining after the production of WAF, CEWAF and dispersant-contaminated seawater and sediment. Overall microbial abundance increased from low control levels to intermediary WAF and dispersant levels and then to high CEWAF levels in both seawater and sediment (Figure 4-2). Differences between treatment was detected in seawater and sediment samples (p-value=0.002 and p-value= 0.004 respectively; Table 4-1). Microbial abundance in control seawater samples were statistically different from all other treatments. Microbial abundance in CEWAF seawater samples were also statistically different from all other treatments (Figure 4-2 and Table 4-2). Microbial abundance in control sediment samples were different from dispersant and CEWAF sediment samples but not from WAF sediment samples (Figure 4-2 and Table 4-2). No statistical differences were found between WAF, CEWAF and dispersant sediment samples (Figure 4-2 and Table 4-2).

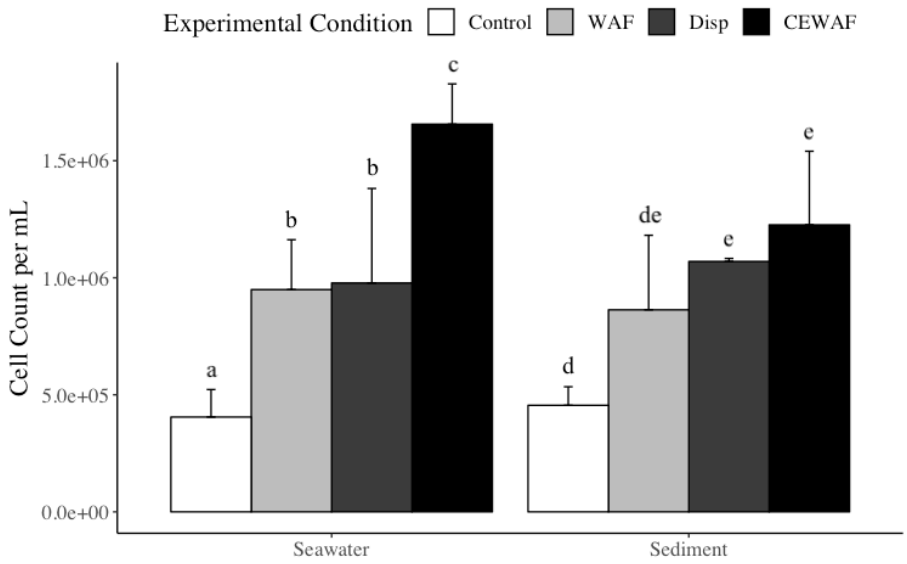

Figure 4-2. Concentrations of prokaryotic cells associated with seawater and sediments after the addition of contaminants. Error bars show the standard deviation of each treatment. Letters highlight statistical differences between treatments.

Table 4-1. Statistical analysis of DAPI staining results across treatments and media. Df is for degrees of freedom. Significant tests are highlighted in bold.

Bartlett Test of Homogeneity of Variances			
	Bartlett's K^2	Df	*p*-value
Seawater	2.73	3	0.44
Sediment	10.83	3	**0.01**
Analysis of Variance			
	F-value	Df	*p*-value
Seawater	12.5	11	**0.002**
One-Way Analysis of Mean			
	F-value	Df	*p*-value
Sediment	42.6	3	**0.004**

Table 4-2. Pairwise t-tests of DAPI staining results across treatments and media. Significant corrected *p*-values are highlighted in bold.

		Control	WAF	Dispersant	CEWAF
Control	Seawater	-	**0.03**	**0.02**	**0.001**
	Sediment	-	0.13	**0.03**	**0.02**
WAF	Seawater	-	-	0.90	**0.02**
	Sediment	-	-	0.36	0.13
Dispersant	Seawater	-	-	-	**0.02**
	Sediment	-	-	-	0.42

4.4.2. Physiology

Respiration rate and clearance rate measurements occurred at the end of the exposure to sediments, to determine the impact of contaminated sediments on *H. panicea* physiology. Respiration rate differed greatly between individual within and across treatments. Overall respiration rate varied from 0.08 µmol per hour per cm^3 of tissue (sample exposed to dispersant contaminated sediments) to 0.37 µmol per hour per cm^3 of tissue (sample exposed to WAF contaminated sediments) (Figure 4-3A). Clearance rate decreased sharply between values measured in control samples compared to values measured in treated samples. Overall clearance rate varied from 0.02 cm^3 of seawater per minute per cm^3 of tissue (sample exposed to dispersant contaminated sediments) to 0.37 cm^3 of seawater per minute per cm^3 of tissue (samples exposed to control sediments) (Figure 4-3B). No statistical differences could be detected between treatment when considering the respiration rate measurements (*p*-value=0.214; Table 4-3). Significant statistical differences were, however, detected between average clearance rates across treatments (*p*-value=0.005). Clearance rates in control samples were significantly higher compared to samples exposed to treated sediments (Figure 4-3B and Table 4-4).

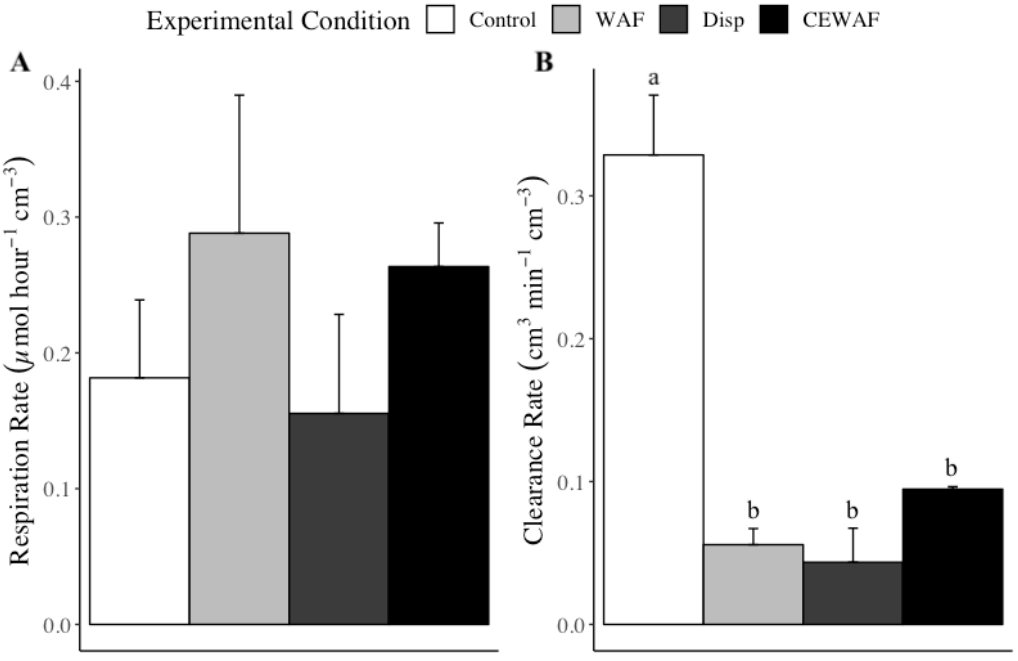

Figure 4-3 Sponge physiological measurements after exposure to contaminated sediments. (A) Respiration rate in (µmol of oxygen consumed per hour per cm^3 of sponge tissue) across treatments. (B) Clearance rate (cm^3 of seawater cleared per minute per cm^3 of sponge tissue) across treatments. Error bars show standard deviation for each treatment. Letters highlight statistical differences between treatments.

Table 4-3. Statistical analysis of physiological measurements. Df is for degrees of freedom. Significant *p*-values are highlighted in bold.

Bartlett Test of Homogeneity of Variances			
	Bartlett's K^2	Df	*p*-value
Respiration rate	0.56	3	0.91
Clearance rate	9.72	3	**0.02**
Analysis of Variance			
	F-value	Df	*p*-value
Respiration rate	1.87	11	0.214
One-Way Analysis of Mean			
	F-value	Df	*p*-value
Clearance rate	34.12	3	**0.005**

Table 4-4. Pairwise t-tests between average clearance rates across treatments. Significant *p*-values are highlighted in bold.

	Control	WAF	Dispersant	CEWAF
Control	-	**4.40E-06**	**3.70E-06**	**1.10E-05**
WAF	-	-	0.56	0.18
Dispersant	-	-	-	0.11

4.4.3. Gene expression

Primers targeting cyclophilin, cytochrome b5 and Hsp70, developed and detailed in chapter 4 were used for gene expression analysis in this study. All three genes were identified as up-regulated after exposure to hydrocarbon contaminated seawater in *H. panicea*. Overall, expression levels for all three genes targeted in this study were highly variable between individuals, leading to strong variations in log fold change (log FC) in all three genes across treatments (Figure 4-4). Control samples exhibited the lowest log FC across all three genes. Cyclophilin and cytochrome b5 log FC between treatment displayed a similar pattern. Control and dispersant treated samples were characterised by low log FC of, respectively, 1.07 ± 0.48 and 1.04 ± 0.22 for cyclophilin and 1.04 ± 0.35 and 0.95 ± 0.44 for cytochrome b5 (Figure 4-4). Cyclophilin and cytochrome b5 appeared up-regulated in WAF and CEWAF samples when log FC reached 2.31 ± 0.91 and 1.85 ± 0.04 for cyclophilin and 2.11 ± 1.34 and 2.15 ± 0.31 for cytochrome b5 (Figure 4-4). In contrast, Hsp70 displayed a different expression pattern. The highest log FC (3.53 ± 2.50) was measured in samples exposed to dispersant contaminated sediments (Figure 4-4). Because of the high variability measured in expression levels between individuals, statistical differences could only be detected when considering cyclophilin (*p*-value=0.03; Table 4-5). Log fold change in control and dispersant samples were found to

be statistically lower than log fold change measured in WAF treated samples (Figure 4-4 and Table 4-6).

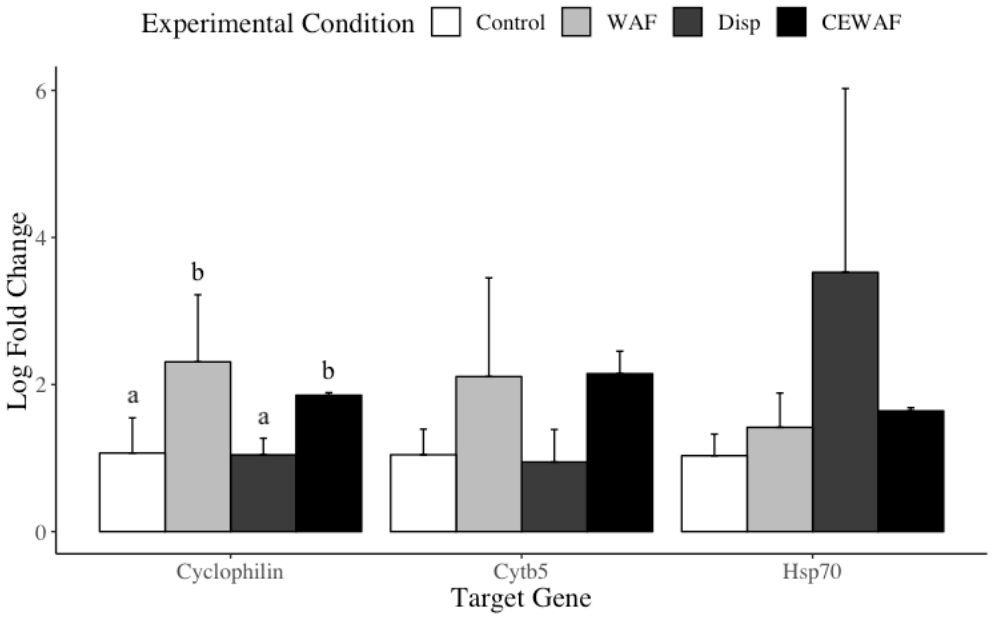

Figure 4-4. Log fold change (log FC) for cyclophilin, cytochrome b5 (cytb5) and heat shock protein (Hsp) 70 across treatments. Error bars show standard deviation for each treatment. Letters highlight statistical differences between treatments.

Table 4-5. Statistical analysis of gene expression data. Df is for degrees of freedom. Significant *p*-values are highlighted in bold.

Bartlett Test of Homogeneity of Variances			
	Bartlett's K^2	Df	*p*-value
Cyclophilin	10.20	3	**0.02**
Cytochrome b5	5.23	3	0.16
Hsp70	17.23	3	**6.30E-04**
Analysis of Variance			
	F-value	Df	*p*-value
Cytochrome b5	2.33	11	0.15
One-Way Analysis of Mean			
	F-value	Df	*p*-value
Cyclophilin	11.12	3	**0.03**
Hsp70	3.58	3	0.14

Table 4-6. Pairwise t-tests between log fold change in cyclophilin expression across treatments. Significant *p*-values are highlighted in bold.

	Control	WAF	Dispersant	CEWAF
Control	-	**0.031**	0.78	0.95
WAF	-	-	**0.031**	0.32
Dispersants	-	-	-	0.95

4.4.4. MiSeq™ sequencing

MiSeq™ sequencing of sponge DNA extractions was performed to study changes in *H. panicea* associated bacterial community after exposure to WAF-contaminated sediments. In control samples, bacteria of the classes γ-Proteobacteria (Alcanivoracaceae), α-Proteobacteria (Rhodospirillaceae, Rhodobacteraceae, Hyphomonadaceae) and Flavobacteria (Flavobacteriaceae) constituted the bacterial community associated with *H. panicea* (Figure 4-5). Significant changes in the relative abundance of bacterial families from control samples were identified in WAF treated sponges. In the first WAF treated sample, previously undetected families, constituting a significant part of the bacterial community, were identified, including Colwelliaceae, Oceanospirillales, Pelagibacter and unclassified α- and γ-Proteobacteria (Figure 4-5). However, in WAF treated samples 2 and 3, a decrease in bacterial family evenness can be seen with Alcanivoracaceae accounting for the majority of the bacteria present in *H. panicea* samples (Figure 4-5).

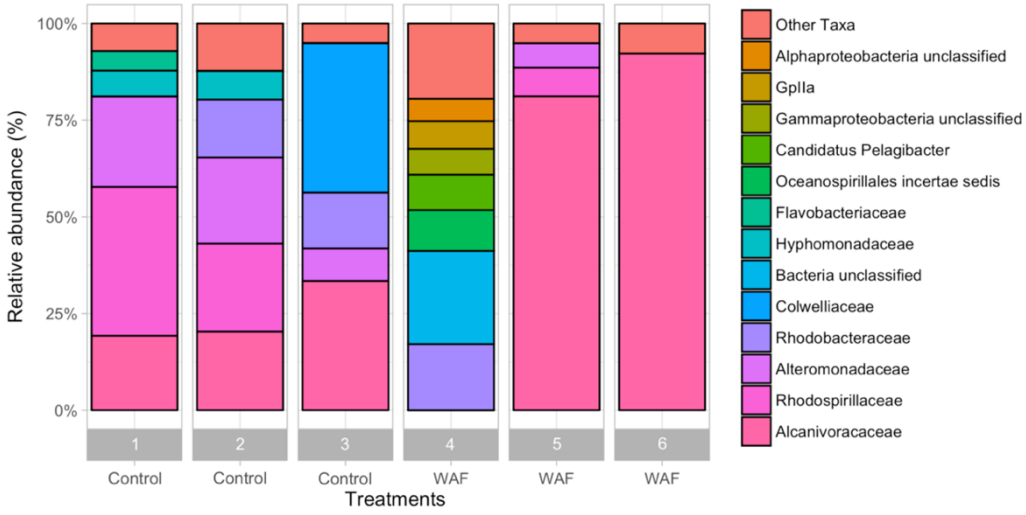

Figure 4-5. Relative abundance of bacterial families in samples exposed to control or water accommodated oil fraction (WAF) sediments.

Rarefaction curves showed that for both control and WAF samples, saturations of sequencing were reached (Figure 4-6). Furthermore, differences in saturation levels confirmed observations made with figure 4-5. OTUs richness levelled at 120 observed OTUs in the control samples where as it only reached 70 in the WAF treated samples (Figure 4-6).

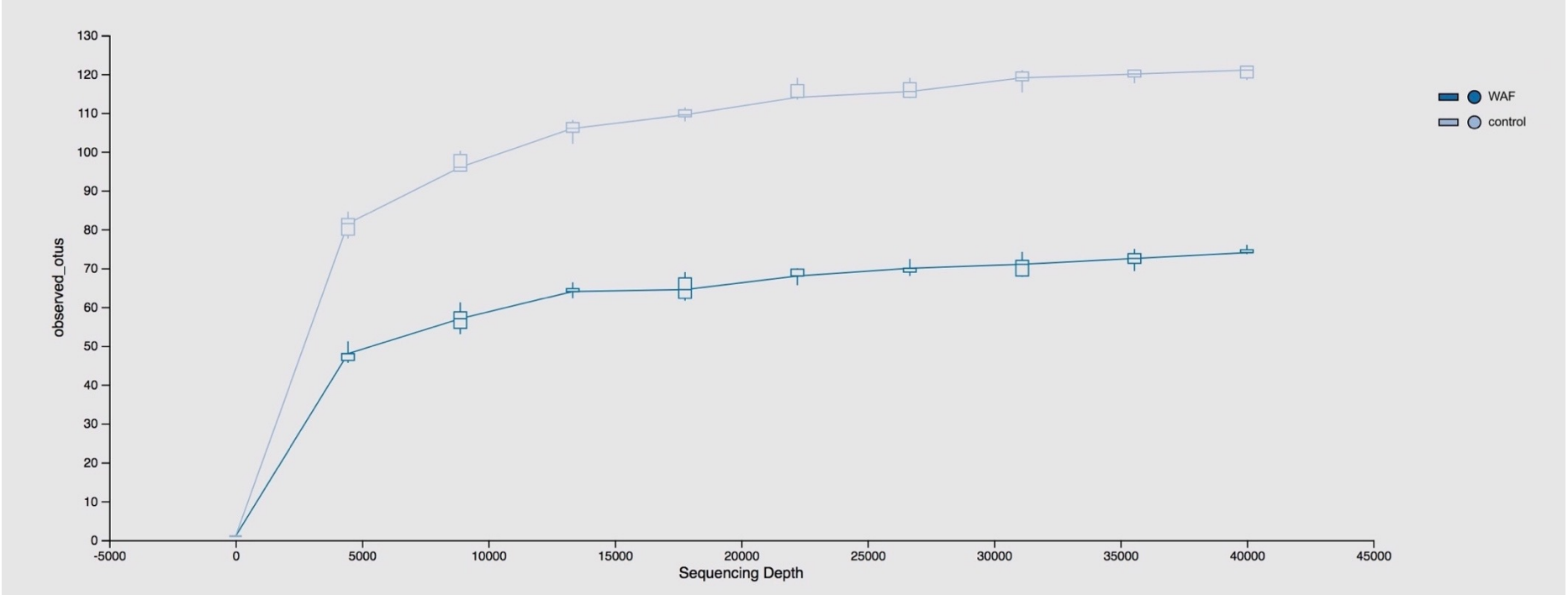

Figure 4-6. Operational taxonomical units (OTUs) rarefaction curves for control and water-accommodated oil fraction (WAF) sediment exposed samples. The number of OTUs is given as a function of the number of reads. Error bars show standard deviation.

4.5. Discussion

4.5.1. Impacts of crude oil, dispersed crude oil and dispersant on seawater and sediment associated prokaryotic communities

Abundance of prokaryotes increased in sediment samples contaminated with crude oil, dispersed crude oil or dispersants. This is in accordance with scientific investigations in cold-water environments (Bunch, 1987; Duran Suja *et al.*, 2017) or with recent literature studying the impact of the Deepwater Horizon oil spill (Kostka *et al.*, 2011; Kleindienst *et al.*, 2015). In WAF, CEWAF or dispersant-contaminated seawater from both the Faroe-Shetland channel and the Gulf of Mexico, laboratory studies have shown that prokaryotic concentration increases significantly, shortly after the addition of the hydrocarbons or the dispersant and stays elevated for weeks (Kleindienst *et al.*, 2015; Duran Suja *et al.*, 2017). Furthermore, in sediments, measurements from field samples both in the artic environment as well as in the Gulf of Mexico showed a proliferation of bacterial cells in oil-contaminated samples (Bunch, 1987; Kostka *et al.*, 2011). In both media (seawater and sediments), the increase in prokaryotic concentration was owing to a bloom of oil-degrading bacteria identified through sequencing techniques (Kostka *et al.*, 2011; Kleindienst *et al.*, 2015; Duran Suja *et al.*, 2017). In this study, DAPI staining only was used to quantify the concentration of prokaryotic cells in both seawater and sediment samples. It is therefore only possible to hypothesise that oil-degrading bacteria are also responsible for the increase in cell concentrations observed.

4.5.2. Physiological impacts of contaminated sediments on *Halichondria panicea*

Exposure to WAF, CEWAF or dispersant contaminated sediments did not appear to impact *H. panicea* respiration rate. Respiration rate values measured in this study are in accordance with the available literature on *H. panicea* despite the high inter-individual variability observed (as reviewed by Osinga *et al.*, 1999). In chapter 3, no difference in respiration rate was detected in sponges exposed to hydrocarbons compared to control measurements. High inter-individual variability in respiration rate was also observed across all tested treatments. Findings from this chapter are therefore in agreement with the results discussed in chapter 3.

Clearance rate appears to decrease in *H. panicea* samples exposed to contaminated sediments. Clearance rate values measured in this study are in accordance with the literature available for other encrusting sponges (De Goeij *et al.*, 2008a). A decrease in clearance rate was also detected, in chapter 3, in *H. panicea* samples treated

with hydrocarbon-contaminated seawater. It appears that trends in physiology results found in this chapter are in accordance with findings from chapter 3. Furthermore, mussel filtration rates have also been described in the literature to decrease after exposure to crude oil-contaminated sediments (Culbertson *et al.*, 2008). Ceasing of the filtration activity could constitute an efficient short-term strategy in filter-feeding organisms to protect their tissue against toxicants. Long-term effects of a decreased filtration rate, and consequently a lowered energy input, should however be studied by testing the impact on sponge survival of longer exposures to contaminated sediments.

Respiration and clearance rates in all conditions, including control, did appear to be overall lower than values gathered in the study presented in chapter 3. Two explanations could contribute to these differences. First, samples collected for this study were collected in the month of November whereas the study presented in chapter 3 was conducted over the summer months. The seawater temperature was notably colder in November and the cold-room for this experiment was set 3° C colder in this experiment (7° C instead of 10° C). Changes in temperature have been shown to impact *H. panicea* physiology (Barthel, 1988) and colder water temperature could therefore contribute to the differences observed here. Second, the resuspension of control sediments could have impacted *H. panicea* physiology. The effects of sedimentation on sponges have been previously investigated as mentioned in chapter 1 and are found to be species specific. Some studies have shown that increase sedimentation can cause a decrease in sponge respiration rate (Tjensvoll *et al.*, 2013; Kutti *et al.*, 2015) and in sponge filtration rate (Tompkins-Macdonald and Leys, 2008), which is in accordance with the findings of this chapter.

A study published in 1989 investigated the impact of sedimentation on *H. panicea* during which the sponge would demonstrate signs of tissue sloughing (Barthel and Wolfrath, 1989). When kept in a flow-through aquaria without particulate filtering (so exposed to sediments), the outer tissue layer of *H. panicea* was completely replaced within two weeks. When the samples were kept in a closed system with no sedimentation, the sloughing events halted (Barthel and Wolfrath, 1989). It is believed that sloughing of the outer tissue layer is a strategy of *H. panicea* to prevent surface fouling (Barthel and Wolfrath, 1989). In the experiment conducted here, no sign of sloughing was found. All samples, across treatments, appeared healthy at the end of the exposure and the outer layer of the sponge did not appear altered in any way. Exposure to sediment only lasted 48 h and this was probably too short to observe any external tissue changes.

4.5.3. Molecular impacts of contaminated sediments on *Halichondria panicea*

Cyclophilin, cytochrome b5 and Hsp70 appeared to be upregulated in the treatment. The genes selected in this study were used because they were found to be upregulated in *H. panicea* after exposure to hydrocarbon-contaminated seawater (chapter 3). Furthermore, all three target genes are known to be involved in hydrocarbon detoxification processes and cellular stress responses in various organism. Cyclophilin play a significant role in a wide range of molecular functions, including in cell signalling (Wang and Heitman, 2005), and was found to be upregulated in the plant model *Arabidopsis thaliana* after exposure to PAH (Liu *et al.*, 2014). Cyclophilin was also upregulated in adult and larvae sponge *Rhopaloeides odorabile* exposed to thermal stress (Webster *et al.*, 2013). Cytochrome b5 is known to influence the cytochrome P450 pathway (Porter, 2002; Stiborová *et al.*, 2014) and was upregulated in oysters exposed to hydrocarbons (Boutet *et al.*, 2004). Hsp70 plays a significant role in regulation of protein translocation processes and protects against cellular damage by preventing accumulation of denatured proteins (Sheffield *et al.*, 1990). Hsp70 has been described to be upregulated in sponges exposed to PCBs (Wiens *et al.*, 1998), cadmium (Schröder *et al.*, 1999) and thermal stress (Lopez-Legentil *et al.*, 2008; Guzman and Conaco, 2016). Hsp70 was also found to be upregulated in the clam *Ruditapes philippinarum* after exposure to BaP (Liu *et al.*, 2015).

Different trends in gene expression profiles of the three target genes were, however, observed. Cyclophilin and Hsp70 seemed to be upregulated in WAF and CEWAF treated samples whereas Hsp70 only appeared to be upregulated in dispersant treated samples. Laboratory studies have shown that dispersants alone can trigger cellular stress responses in a range of organisms (Venn *et al.*, 2009; Hook and Osborn, 2012; Zhang *et al.*, 2013). Exposure to dispersant Corexit 9527 led to upregulation of Hsp70 in coral *Montastraea franksi* (Venn *et al.*, 2009). Findings from this study are, therefore, in accordance with the available scientific literature. This is the first study to demonstrate the effects of dispersant contaminated-sediments on sponge gene expression patterns. As dispersant can persist in sediments for months after an oi spill (White *et al.*, 2014), results from this chapter present evidences for potential long-term molecular stress in sponges when exposed to dispersant contaminated sediments. As highlighted in chapter 3, the use of dispersant in areas of high sponge density should, therefore, be avoided or if not possible, carefully considered.

Nevertheless, it is important to emphasise that high variations in log fold change between individuals across treatments were observed. Consequently, only one statistically significant difference was detected for cyclophilin between control and WAF treated samples. Tissue sampling in this study occurred at the end of the exposure (48 h). In contrast, samples collected for transcriptomic analysis were collected during the exposure, approximately 24 h after the start of the exposure (chapter 4). This could explain the lack of statistical differences found. Further studies determining the gene expression profiles over time should be conducted to resolve when samples should be collected in *H. panicea* ecotoxicology experiments.

4.5.4. Composition of the *Halichondria panicea* associated microbial community and impacts of crude oil contaminated sediments

Overall, control samples in our study were characterised by a homogenous bacterial community constituted by a high OTU diversity. Five bacterial families constitute the majority of the microbial community associated with control *H. panicea* samples: Alcanivoracaceae, Rhodospirillaceae, Alteromonadaceae, Rhodobacteraceae and Colwelliaceae. Althoff *et al.* (1998) demonstrated that Rhobacteraceaea constituted the dominant bacterial family of the microbial community of *H. panicea* samples collected from the North Sea. Furthermore, another study on *H. panicea* associated microbial community showed that *Rosebacter* also belonging to Rhodobacteraceaea was predominant (Wichels *et al.,* 2006). The bacterial community described in my samples are therefore very different from the profiles given in earlier studies (Althoff *et al.,* 1998; Wichels *et al.,* 2006). Changes to the microbial community in the Coldingham Bay samples could have occurred while the sponges were kept in retention tanks in the lab, prior to the experiment. A field control should have been collected and analysed alongside the experimental sample to detect the impact of the laboratory practice on the sponge associated bacterial community.

The presence of significant levels of Alcanivoracaceae and Colwelliceae in the control samples of this study was also unexpected. Bacteria from the *Alcanivorax* genus, belonging to the family Alcanivoracaceae are known as obligate oil-degraders (Yakimov *et al.*, 2007; Joye *et al.*, 2016). Moreover, Colwellieceaea have been shown to be present in anaerobic conditions in sediments (Bowman and McCuaig, 2003; Nogi *et al.*, 2004; Jung *et al.*, 2006) and sponges (Jackson *et al.*, 2012). Bacteria of the genus *Colwellia* are capable of dispersant and oil degradation (Valentine *et al.*, 2010; Kleindienst *et al.*, 2015). Bacteria of the genus *Alcanivorax* and *Colwellia* were both detected in the succession of

a bacterial bloom observed after the Deepwater Horizon oil spill (Yang *et al.*, 2014). Oil-degrading bacteria have been previously found in sponges, growing next to oil seeps (Arellano *et al.*, 2013; Rubin-Blum *et al.*, 2017). However, this is the first-time oil degrading bacteria have been identified in shallow-water sponges not exposed to a natural source of hydrocarbons.

Little information is known about Coldingham bay in the scientific literature. Contaminant monitoring studies of coastal Scottish waters have shown that East Scotland is characterised by low but detectable concentrations of PAHs (Webster *et al.*, 2011). No specific information on Coldingham bay is available. Unfortunately, no water sample was, collected during this study for the purpose of genomic sequencing. Further studies of the *H. panicea* in Coldingham bay should be conducted to better understand the presence of oil-degraders in the sponges. For example, seasonal sampling of *H. panicea*, Coldhingham bay surface water and sediments could be undertaken as conducted in Jackson *et al.* (2012).

After exposure to WAF contaminated sediments, bacterial communities associated with *H. panicea* shifted towards a specialised oil-degrading community. Overall OTU richness decreased as members of Alcanivoracaceae dominated the community in most samples. One sample, however, did not follow this trend. In this sample, the family Oceanospirillales and Colwelliaceaea, also comprising oil degrading bacteria, were detected. As mentioned in chapter 1, other studies have found that sponge associated microbial communities can change when sponges are exposed to contaminants (Webster and Hill, 2001; Webster *et al.*, 2008; Fan *et al.*, 2013; Tian *et al.*, 2014). This study is the first to show evidence for the bacterial community adapting to the specific contaminant type the host sponge is exposed to. It could be hypothesised that this shift the in microbial community could provide *H. panicea* with a heighten resilience to oil contamination. Further studies into the metabolic activities of oil-degraders in the sponge tissue through metatranscriptomics (Moitinho-Silva *et al.*, 2017) should be conducted to assess this hypothesis.

4.7. Conclusion

Findings from this chapter showed that the clearance rate in *H. panicea* decreased sharply when exposed to WAF, CEWAF or dispersant-contaminated sediments, while respiration rate did not seem to be affected. Changes in expression levels of genes involved in the cellular detoxification of hydrocarbons were also observed. Miseq™ data

demonstrated that *H. panicea* from Coldingham bay harbour oil-degrading bacteria which dominated the microbial community when the sponge host was exposed to WAF treated sediments. As oil and dispersants can persist in sediments for extended periods of time, these results suggest that an oil spill could have long-lasting effects on sponges. Weathering of the oil should, however, be considered here and further investigation on the impact of weathered oil contaminated sediments needs to be conducted. The next chapter will further summarise the findings of this thesis and discuss future work.

Chapter 5 Synthesis and Future Work

5.1. Overview

The overall objective of my PhD project was to determine the impacts of offshore oil and gas activities on sponges throughout the life stages of an oil field and during a potential oil spill. Here, I will summarise the work presented in each chapter. Additional work conducted during the PhD project, presented in appendices C and D will also be discussed. Finally, an overall conclusion and recommendation for future work will be provided.

5.2. Chapter summaries

5.2.1. Chapter 1 - Potential impacts of offshore oil and gas activities on deep-sea sponges and the habitats they form

The aim of chapter 1 was to provide a detailed overview of the impacts of offshore hydrocarbon production activities on deep-sea sponges at multiple biological scales, throughout the lifetime of a field. From this literature review, it appeared that:

(1) The different activities taking place during the consecutive phases of an oil field development (discovery/appraisal, production, decommissioning) can lead to a range of biological impacts on deep-sea sponge grounds as habitats, on individual organisms and/or within their tissue and cells.

(2) While some studies have investigated the impacts of chemical releases occurring during hydrocarbon production on deep-sea sponge species, most surveys have been conducted on either shallow-water sponges or on other filter-feeding organisms.

(3) An oil spill occurring in the vicinity of a deep-sea sponge ground could cause long-lasting biological effects on sponges and their associated fauna, but further experimental work is needed to understand these effects and their long-term biological and ecological implications.

(4) Considerable research gaps persist at all stages of the field development, across biological scales, and further work on the resilience of sponge to oil and gas activities is needed.

5.2.2. Chapter 2 – Influence of environmental and anthropogenic factors on deep-sea megafauna and deep-sea sponges in the Faroe-Shetland channel

The aim of chapter 2 was to determine which environmental factors controlled the spatial distribution of deep-sea sponges and associated megafauna in the Faroe-Shetland channel. Additionally, the impact of demersal fishing activities and hydrocarbon

production activities on the local megafauna was investigated. This work was conducted using seabed surveys collected by the oil and gas industry, academic partners and governmental resources. My data collection and analysis from these surveys showed that:

(1) Substrate characteristics and temperature exert a strong influence on the spatial distribution of megafauna in the Faroe-Shetland channel.
(2) Demersal fishing effort, varying across oil fields within the Faroe-Shetland channel, negatively impacted both sessile and motile megafauna.
(3) Motile megafauna were associated with larger oil and gas infrastructure, away from drilling wells.
(4) Understanding at which spatial scales factors vary can significantly change how they seem to impact community distribution.
(5) Taking all anthropogenic activities into account is important to appropriately estimate their impact on the environment.

Chapter 2 contributed to further understand the impact of oil and gas activities on sponge grounds and provided evidence for the potential impacts of decommissioning oil and gas infrastructures in the vicinity of deep-sea sponge grounds.

5.2.3. Chapter 3 – Impacts of crude oil and dispersed crude oil contaminated seawater on sponge *Halichondria panicea* at physiological, histological and molecular levels

The aim of chapter 3 was to investigate the biological effects of crude oil, dispersed crude oil and Benzo[a]Pyrene (BaP) contaminated seawater on the sponge *H. panicea*. This chapter provided the first study of the impact of water-accommodated crude oil fraction (WAF) and chemically enhanced (CEWAF) on any sponge species and demonstrated that:

(1) While respiration rate did not seem impacted by any of the treatment conditions, clearance rate significantly decreased in *H. panicea* samples exposed to hydrocarbon contaminated seawater.
(2) By increasing the concentration of hydrocarbons in seawater, the dispersant Slickgone NS used in the production of CEWAF, significantly lowered the clearance rate ED50 and led to mortalities at high oil loadings.
(3) Transcriptomic analysis of *H. panicea* samples revealed that a homogenous gene response was triggered after exposure to WAF, CEWAF or BaP alone, although more differentially expressed genes were detected in the CEWAF treatment.

(4) Transcriptomic analysis also showed that most likely the whole holobiont constituted by the sponge and its associated microbial community, was impacted by the presence of hydrocarbons
(5) Transcriptomic analysis of *H. panicea* samples demonstrated that genes involved in the sponge metabolic process as well as its defence response were significantly up-regulated.
(6) Histological investigation of *H. panicea* samples showed that exposure to both WAF and CEWAF lead to the formation of necrotic tissue lesions.

5.2.4. Chapter 4 – Impacts of crude oil, dispersed crude oil and dispersant contaminated sediments on sponge *Halichondria panicea* and its associated microbial community

The aim of chapter 4 was to study the impacts of crude oil and dispersed crude oil contaminated sediments on *H. panicea*. Hydrocarbons and dispersant can potentially reside in sediments for extended period of time owing to slow hydrocarbon degradation processes taking place in anaerobic and nutrient-depleted conditions. This chapter showed that:

(1) Clearance rate decreased in *H. panicea* samples exposed to contaminated sediments, while respiration rate did not appear to change.
(2) No clear up-regulation of target genes cyclophilin, cytochrome b5 and Heat shock protein 70 was detected in samples exposed to contaminated sediments.
(3) MiSeq™ sequencing of DNA extract from *H. panicea* control samples revealed a surprising microbial community composition with the presence of oil degrading bacteria.
(4) A decrease in the sponge associated microbial community diversity was observed in *H. panicea* exposed to WAF contaminated sediments owing to a stark increase in the abundance of oil degrading bacteria.

The work conducted in this chapter, if confirmed, would provide the first example of oil degrading bacteria in a shallow-water sponge not associated to a natural oil seep and could hint at sponge-associated oil-degrading bacteria thriving on hydrocarbons to which the holobiont is exposed.

5.3. Additional work conducted during the PhD project

Chapters 3 and 4 of this thesis presented experimental work conducted on intertidal shallow-water sponge *H. panicea*, put forward in this thesis, as an ideal model sponge species for experimental studies. Comparisons with other sponges is however

needed to determine if the responses of *H. panicea* are found in other species. For example, it is possible that its intertidal habitat may make *H. panicea* relatively resilient to environmental stressors compared to deep-water species adapted to a far less changeable environment. Furthermore, as many different WAF and CEWAF protocols are available in the literature, it is important to test if the protocols used here could also impact the outcome of WAF/CEWAF exposure experiments. Appendix C present experimental work conducted on *Myxilla sp*, developed to answer these questions. A comparison between the responses of the two sponge species is provided as well as a comparison between the CROSERF and the Kleindienst protocols (Aurand and Coelho, 2005; Kleindienst *et al.,* 2015). In summary, preliminary experimental work shows that at physiological level, *Myxilla sp* respond in the same way as *H. panicea*. Furthermore, no differences in the sponge response to exposure to WAF could be detected when comparing the CROSERF and Kleindienst protocols (Aurand and Coelho, 2005; Kleindienst *et al.,* 2015).

One of the initial objectives of the thesis was to ultimately conduct experimental work on deep-sea sponges. This was not achieved due to external circumstances. However, coastal regions can also be at risk of oil spills and studying the resilience of shallow-water sponges is, therefore, in itself a relevant scientific question, especially in areas where the local coastal biota is poorly known. Greenland is expected to hold a vast amount of undiscovered hydrocarbon deposits (Harsem *et al.,* 2011). Licences for hydrocarbons have recently been allocated, including in areas offshore Nuuk, southwest Greenland (Hurup and Hansen, 2014). Increased commercial shipping, owing to the opening of the Northwest Passage as a result of global warming, is also of a concern (Harsem *et al.,* 2011). Yet, Greenlandic coastal environments are still very poorly known (Jensen and Christensen, 2003). In the summer 2016, a series of diving surveys around Nuuk were organised by Dr K. Schoenrock (U. of Glasgow, now NUI Galway) and Dr N. Kamenos (U. of Glasgow), initially to investigate kelp forests and maerl bed habitats biodiversity (see appendix A for publication). Sponge collection was also undertaken during these dives. Two sets of exposure experiments were carried on different sponge species and additional samples were preserved for identification purposes. The objective of the study was to contribute to the development of an environmental baseline for Greenlandic coastal environments. Preliminary results of this work are presented in appendix D.

5.4. Conclusion

The work undertaken during my PhD project combined different type of studies ranging from significant dataset collection and analysis (chapter 2) to experimental investigations (chapters 3 and 4). Techniques including advanced spatial statistical analysis, physiological data gathering, histological investigation and sequencing were applied. Effects of oil and gas activities are on habitat level (chapter 2) as well as individual and cellular/molecular levels (chapter 3 and 4) were identified. Contributions from this body of work to the wider scientific knowledge can hence be added to the summary table presented in table 1-2 (Table 5-1). Based on the work presented in this thesis, the following recommendations for the protection of deep-sea sponge and sponge grounds from anthropogenic activities can be given:

(1) Spatial footprint of infrastructure at the seabed should be limit within sponge grounds.
(2) Fishing activities should be limited within sponge grounds.
(3) The use of dispersants in oil spill remediation responses should be avoided or, if not possible, strongly reconsidered, within sponge grounds.
(4) Experimental work on deep-sea sponge species should be supported by the oil and gas industry to fully clarify the impact of hydrocarbon and/or dispersant exposure on these vulnerable organisms.

At habitat level, the impacts of human activities from different industry sectors can lead to contradictory effects on sponge grounds. In the Faroe-Shetland channel, megafauna distribution was mostly controlled by substrate characteristics and temperature. However, the presence of oil and gas infrastructure removed from the well sites seemed to provide a suitable habitat for many benthic organisms, shielded from the fishing activities. The spatial scale of surveys and consideration on spatial autocorrelation is of primary importance here but is often overlooked in deep-sea community studies. In the future, the use of automated underwater vehicles (AUVs), able to gather environmental data over large areas (Morris *et al.*, 2014), could constitute an answer to this issue. AUV use could also facilitate the monitoring over time (years) of the Faroe-Shetland channel nature conservation marine protected area. A management plan proposal was issued in 2017, limiting fishing activities within the nature conservation marine protected area (Marine Scotland, 2017ab). Only temporal data could reveal if management plans are successful in protecting deep-sea sponges from human activities and surveys over decades will be needed.

Research in oil spill remediation other than dispersants needs to become a priority. Exposure to hydrocarbons led to a significant and sustained decrease in filtration activity. The use of dispersant worsened the scale of the sponge response and resulted in mortality at high hydrocarbon concentrations. The use of dispersant in oil spill remediation strategies is under great discussion in the scientific literature (John *et al.*, 2016; Nyankson *et al.*, 2016). Alternative remediation strategies are also being investigated including the use of biologically-derived dispersant (bio-dispersants) (Gutierrez, 2017) or bioremediation (addition of nutrient or microorganisms to seawater in the area of a spill) (Swannell *et al.*, 1996; Crisafi *et al.*, 2016). The impact of such remediation strategies should be investigated on benthic organisms such as sponges.

Sequencing technologies (MiSeq™ and transcriptomic) showed that exposure to hydrocarbon led to prominent changes in the host gene expression profile and in the symbiont microbial community composition. These findings contribute to enhance the understanding of how the sponge-associated microbial community changes within the host and how it could adapt to external stressors. Further work is, however, needed to determine changes to metabolic processes in the bacterial community in response to external contaminants. Metatranscriptomic and metaproteogenomic constitute two approaches that could be used, to partly answer this question (Liu *et al.*, 2012; Moitinho-Silva *et al.*, 2017; Slaby *et al.*, 2017).

5.5. Future work

The transcriptomic data gathered during this PhD project offers opportunities for further studies. From the sequencing data now available for *H. panicea*, a series of stress related genes could be selected, and primers sets for each target genes could be developed. This approach was already applied in this thesis for three target genes (cyclophilin, cytochrome b5 and Hsp70) but could be expanded to a set of eight to ten genes. This could allow *H. panicea* to be used in series of ecotoxicology experiments, including to understand the impact of temperature change on a temperate shallow-water sponge, so far only studies in tropical sponge *Amphimedon queenslandica* (Pantile and Webster, 2011; Fan *et al.*, 2013). After selection of the target genes, determining the temporal response of each of these genes after exposure should be a priority, to understand how quickly genes respond in *H. panicea*.

Table 5-1: Updated summary table presented in chapter 1. Impacts investigated in deep-sea sponges are highlighted in green. Impacts described in shallow-water sponges but not yet confirmed in deeper species appear in orange. Impacts found in other benthic organisms and not yet investigated in any sponge are marked in red to emphasize current knowledge gaps. Contributions from this thesis have been highlighted in bold and underlined font.

		Exploration and appraisal	**Field Development**	**Production**	**Decommissioning**	**Deep-sea oil spill**
Community level	*Main concern*	*Physical disturbance of seabed and increase sedimentation*		*Discharge of drill muds and cuttings*	*Removal of structure*	*Exposure to high hydrocarbons and dispersant concentrations*
	Impacts					Changes in benthic community abundance, age structure and trophic interactions.
		<u>Habitat provisioning at large spatial scales in fished areas.</u>			**<u>High probability of habitat destruction.</u>**	**<u>High probability of sponge grounds being impacted due to the closeness to oil and gas infrastructures.</u>**
		Diminished benthic community.		Benthic community diversity/abundance decrease.		
Individual Level	*Main concern*	*Seismic survey increase sedimentation*	*Increase sedimentation*	*Discharge of produced water*	*Release of chemical contaminants*	*Exposure to high hydrocarbons and dispersant concentrations*
	Impacts	Larval development delay and malformations.				Health decline, hydrocarbon bioaccumulation.
		Changed respiration rate and reproduction capacities. Decreased growth rate.		Bioaccumulation of PAH and heavy metals.		Larval settlement disturbance. Hydrocarbon bioaccumulation. **<u>Sustained decrease in clearance rate. Lethal effects of dispersed crude oil.</u>**
		Paused filtration.				
Cellular & Molecular levels	*Main concern*	*Discharge of drill muds and exposure to chemicals via release of produced water*				*Exposure to high hydrocarbons and dispersant concentrations*
	Impacts	Decrease immune system function.				
		Activation of MAPKs and cytochrome P450 pathways. Oxidative stress.				**<u>Activation of MAPKs and cytochrome P450 pathways. Oxidative stress. Impacted immune system. Tissue lesion. Change in associated microbial community</u>**
		Decrease of lysosomal membrane stability.				

Variations of the experiments conducted during my PhD project could help further determine the resilience of *H. panicea* to crude oil and dispersed crude oil contamination. Specifically, testing the resistance of *H. panicea* to longer exposure time would be of particular interest. While sponges may be able to survive an exposure time of 48 h by ceasing their filtration activities, surviving a longer exposure time might be difficult. Furthermore, MiSeq^{TM} data from chapter 4 seems to establish evidence for the presence of oil-degrading bacteria within *H. panicea,* collected at Coldingham bay. The role that oil-degrading bacteria plays in the resilience *of H. panicea* to oil contaminated media should be further explored. This could be achieved by the use of metatranscriptomic sequencing already applied in sponges in several studies (Moitinho-Silva *et al.*, 2017). Finally, exposure to weathered oil contaminated seawater/sediments should also be considered. The chemical characteristics of crude oil changes significantly through time, which in turn could modify the response of *H. panicea*.

In this thesis, two crude oil exposure pathways were investigated: through contaminated seawater (chapter 3) and through resuspension of contaminated sediments (chapter 4). A third important exposure pathway should also be considered: namely through the consumption of marine oil snow (MOS). MOS is constituted by floating organic particles of less than 0.5mm in diameter, oil droplets and mucus (Duran Suja *et al.*, 2017). MOS formation and subsequent sedimentation was studied during the DeepWater Horizon oil spill (Valentine *et al.*, 2014). However, the impact of MOS to benthic filter-feeding organisms, such as sponges still needs to be investigated (Van Eenennaam *et al.*, 2018). MOS has successfully been recreated in laboratory conditions (Duran Suja *et al.*, 2017) and feeding experiments, using *H. panicea*, could therefore now be conducted.

As mentioned earlier, experimental work on deep-sea sponge species is needed to determine their resilience to crude oil and dispersed crude oil exposures. Deep-water organisms, often characterised by a slower growth rate, a long lifespan and late reproduction, are considered to be less resilient to change in their environment than shallow-water species (Armstrong *et al.*, 2012). Deep-sea sponge grounds are designated as vulnerable marine ecosystems (United Nations Food and Agriculture Organisation) and ecologically and biologically significant areas (United Nations Convention on Biological Diversity) (Hogg *et al.*, 2010). Sponge grounds constitute important biodiversity hotspots in the deep sea. Areas dense in deep-sea sponges are continuously discovered within areas of interest to the oil and gas industry (Amon *et al.*, 2017; Hajdu

et al., 2017; Williams *et al.*, 2018). Conducting ecotoxicological experiments on deep-sea sponges is, therefore becoming more and more important, as it can be hypothesised that lethal effects could be detected at lower concentration of contaminants.

Appendix A

List of Publications

1. Publications adapted from chapters of this thesis

Vad, J., Kazanidis, G., Henry, L.-A., Jones, D.O.B., Tendal, O. S., Christiansen, S., Henry, T. B. and Roberts, J.M. (2018) 'Potential Impacts of Offshore Oil and Gas Activities on Deep-Sea Sponges and the Habitats They Form', *Advances in Marine Biology*, 79, pp. 33-60. doi: 10.1016/bs.amb.2018.01.001.

Vad, J., Kazanidis, G., Henry, L.-A., Jones, D.O.B., Gates, A.R. and Roberts, J.M. SUBMITTED 'Environmental Controls and Anthropogenic Impacts on Deep-Sea Sponge Grounds in the Faroe-Shetland Channel, NE Atlantic', *Scientific Reports*.

Vad, J., Dunnett, F., Liu, F., Montagner, C., Roberts, J.M. and Henry T.B. IN PREP 'Soaking Up the Oil: Biological Impacts of Crude Oil and Dispersed Crude Oil on *Halichondria panicea*', *Marine Pollution Bulletin*.

Vad, J., Duran Suja, L., Summers, S., Roberts, J.M. and Henry T.B. IN PREP 'Effects of Crude Oil, Dispersed Crude Oil and Dispersant Contaminated Sediments on Model Sponge *Halichondria panicea*', *Marine Ecology Progress Series*.

2. Other publications derived from work conducted during the PhD project

Vad, J., Orejas, C., Moreno-Navas, J., Findlay, H.S. and Roberts, J.M. (2017) 'Assessing the Living and Dead Proportions of Cold-Water Coral Colonies: Implications for Deep-Water Marine Protected Area Monitoring in a Changing Ocean', *PeerJ*, 5, p.e3705. doi: 10.7717/peerj.3705.

Schoenrock, K.M., Vad, J., Muth, A., Pearce, D.M., Rea, B.R., Schofield, J.E. and Kamenos, N. (2018) 'New Baselines: A Description of Kelp Forests and Coralline Algae Habitats in Southwestern Greenland', *Diversity,* 10(4):117.

Kazanidis, G., Vad, J., Henry, L.-A., Neat, F., Berx B. and Roberts J.M. SUBMITTED 'Distribution of Deep-Sea Sponge Aggregations and the Role of Environmental Variability in the Faroe-Shetland Channel', *Frontiers in Marine Science*.

3. Publications originating from collaborations

Mackenzie, C.L., Vad, J. and MacPherson, R. (2018). Clarification of Governance Relevant to the Sustainable Management of Marine Species and Habitats within the United Kingdom: An Overview of Regional, National and International Authorities, Advisories, Legislation and Designation Types with Summary Schematic Tool. *Environmental management*, pp.1-13. doi: 10.1007/s00267-018-1064-z.

Duran Suja, L., Vad, J., Henry, L.-A. and Roberts, J.M. IN PREP 'The Tunicate *Polycarpa pomeria* (Savigny, 1816), a Possible Contributor to Cold-Water Coral Reef Stability and Ecosystem Function' *Heliyon*.

Appendix B

Experimental Method Development Conducted with *Halichondria panicea*

1. Overview

This appendix describes the results from the preliminary experiment conducted on *Halichondria panicea* when the experimental protocol used in the thesis was developed. Sponges were exposed for 48h to water-accommodated oil fraction (WAF), chemically enhanced WAF (CEWAF), dispersant contaminated seawater (Dispersant) and control seawater. Physiology measurements as well as COMET assay were then carried out at the end of the exposure. This experiment was conducted using simple incubation chamber lacking internal mixing and was therefore not considered in the main section of the thesis. Results were used to determine which biological endpoints to use in further experimental work. Aquaria husbandry developed for the rest of the experimental work is also discussed in this appendix.

2. Material and Methods

Sponge and seawater sampling

As explained in chapter 3 and 4, *H. panicea* samples were collected at Coldingham bay, located 80 km to the south of Edinburgh, Scotland. Sponges were carefully removed with a scalpel from the rocks and placed into sampling bags filled with freshly collected seawater. Surface seawater was also collected from Coldingham bay. Sponge samples were initially split between St Abbs marine Station, Heriot-Watt University and Edinburgh University for aquaria husbandry testing (see next subsection).

Aquarium husbandry

Prior to any experimental work, trials to maintain *H. panicea* samples in aquaria at St Abbs marine station, Heriot-Watt University and the University of Edinburgh were conducted. Different installations were available at each institution and sponges had not been previously kept at any of these locations. St Abbs marine station is located 76 km from Edinburgh but only 4 km from Coldingham bay which offers the advantage of limiting transportation time (and stress to the sponges) after sampling. St Abbs is also equipped with a flow-through system that circulates seawater from the bay and through tanks. Tanks are kept outside (under a covered transparent canopy) and are therefore exposed to natural temperature, nutrients and luminosity variations. However, this set-up does not allow for experimental work to be conducted at the marine station as pollutants would be released back into the marine environment. If kept at St Abbs, samples would therefore require to be transported back to Edinburgh for experimental work. At Heriot-

Watt University, sterile seawater was readily available, and sponges were kept in a shared cold-room facility. Sponges were therefore kept in a controlled environment and fed with *Isochrysis* Instant Algea® (Reed Mariculture, California) diluted solutions. At the University of Edinburgh, only artificial seawater, prepared with reverse osmosis water and Tropic Marin® salt, was available. However, sponges could be kept in a separate cold-room, not in use at the time for any other experimental work. Here, sponges were also kept in a controlled environment and fed with *Isochrysis* Instant Algea® (Reed Mariculture, California) diluted solutions. After a few weeks of cultivation time, samples at all three locations were visually inspected and all samples seemed to remain healthy: they retained their bright yellow colours and their outer tissue layer remained unchanged. No differences between the three locations was found and it was therefore decided to keep sponges at Heriot-Watt University and the University of Edinburgh where experimental work could be easily conducted. For this experiment, sponges were kept at Heriot-Watt University.

At each university, prior to experimentation, sponges were kept in two retention tanks of 35 L in volume. The retention tanks were connected to two sumps of 200 L which allowed for seawater/artificial seawater to be continuously circulated through the retention tanks. Air was bubbled into the sump to allow oxygenation of the seawater/artificial seawater. One third of the seawater from the sump was changed every week and salinity was monitored and kept at 35 ppm. As mentioned above, sponges were fed every two days by adding a few millilitres of *Isochrysis* Instant Algea® (Reed Mariculture, California) solution. The tanks were kept at 8-10° C.

Trial incubation chambers

In order to conduct the initial experimental trials on *H. panicea* and presented in this appendix, twelve trial incubation chambers were constructed. These glass chambers had a metallic screw-on lid through which glass inflow and outflow tubes were installed. These chambers were not equipped with a stirring mechanism and mixing in the chamber was only achieved through the movement of the water pumped in at the bottom of the chamber and flowing out at the top of the chamber.

Two Marine Color™ peristaltic pumps were used to circulate the water through the chambers. Each chamber held a volume of 250 mL and were connected through PTFE tubing to the peristaltic pumps. Each channel of the peristaltic pumps was itself connected back to a one litre Duran® bottle containing the relevant treatment solution. Air bubbling was provided into each Duran® bottle through PVC tubing connected to glass Pasteur

pipettes so that the treatment solutions were only in contact with the glass pipettes. Flow was set at 500 mL per 24 h to allow the water in the trial chambers to be fully replaced twice per day. Some of the results obtained in the preliminary experiments using the trial incubation chambers are presented in the next sections. These simple incubation chambers were, however, not used in any other experiments because of the lack of internal mechanical mixing. Due to this design flaw, the sponges had to be transferred into separate respiration chambers at the end of the experimental run to allow for respiration and clearance rate measurements to be conducted properly (as explained below). More advanced incubation chambers were therefore designed to improve the experimental work, as presented in chapters 3 and 4.

The Kleindienst et al. protocol (2015)

The Kleindienst protocol was used in the initial experiment conducted on *H. panicea* and presented in this appendix. As explained in chapter 4, this protocol is based on the preparation of stock solutions of WAF and CEWAF that are then diluted into working solutions of WAF and CEWAF. For the preparation of one litre of WAF stock solution, 0.15 L of crude oil was mixed with 0.85 L of sterile seawater. 0.015 L of dispersants was added to the mixture for the preparation of CEWAF stock solution. The mixture was then mixed for 48 h. After mixing, the stock solutions were left to rest for one hour and 0.4 L of water fractions from the stock solutions were carefully removed from the mixture and added to 1.4 L of unfiltered seawater to obtain the WAF and CEWAF working solutions.

Several publications have called into question the protocol and conclusions explained in Kleindienst *et al.* (2015) (Lewis, 2015; Kleindienst *et al.*, 2016; Prince *et al.,* 2016). Appendix C provide preliminary results of comparative experimental work conducted on *Myxilla sp* with both protocols.

Sponge volume determination

The volume of each sponge sample, to be used for the normalisation of the respiration and clearance rate, was determined at the end of the preliminary experiment using the same method as described in chapters 3 and 4. The thickness of the sample was measured directly with a calliper. A photography was then taken of each sample and the freely available software Fiji was used to determine the surface area of the sample (Schindelin *et al.,* 2012). Surface area was then multiplied by the thickness to determine the sponge volume.

Respiration rate

Respiration rate was determined by analysing the decrease in O_2 over time in each chamber, at the end of the exposure, as explained in chapters 3 and 4. Concentration of O_2 in each chamber was measured every 15 seconds during two hours using Presens sensor spots connected to Oxy-4 optodes (Presens Precision Sensing GmbH, Germany). A two-hour time period was enough to detect a significant change in O_2 concentration in the respiration chamber. Stirring in the chambers was kept active during the measurement time but the inflow and outflow were closed so no fresh input of seawater was added. To account for microbial respiration in the seawater, blank measurement of respiration in seawater and treatment solutions were also determined in empty chambers. $\%O_2$ was converted to µmol/L using the R package presens (Birk, 2016). Sponge respiration rate were then determined using the following formula:

$$Respiration\ rate = (Resp_{Chamber} - Resp_{blank})/V_{sponge}$$

where $Resp_{chamber}$ is the respiration rate determined in the chamber with a sponge, $Resp_{blank}$ is the respiration rate determined in the blank chamber without a sponge and V_{sponge} the volume of sponge tissue (determined at the end of the experiment).

Clearance rate

To determine clearance rate, *Isochrysis* Instant Algea® (Reed Mariculture, California) diluted solution was added to each chamber and the sponges were left to filter for two hours. Stirring in the chambers was kept active during the measurement time but the inflow and outflow were closed so no fresh input of seawater was added. Water samples were collected every 20 minutes and algae cell concentrations were determined through total absorbance measurements. Clearance rates for each sample was calculated as follows (De Goeij *et al.*, 2008b):

$$Clearance\ rate = (\left(\frac{V_{water}}{t}\right) \ln\left(\frac{C_0}{C_t}\right))/V_{sponge}$$

where V_{water} is the volume of water in the chambers, t the time of incubation, C_0 and C_t the initial and final concentration of algae in the chamber and V_{sponge} the volume of sponge tissue.

Tissue sampling

Tissue samples were collected at the end of the experiment. About 3 mm^3 of tissue was cut from individual sponge samples with a sterile scalpel and thoroughly rinsed with MilliQ™ water to remove any outer debris and seawater. Samples were then either used immediately for COMET assay.

Single-cell electrophoresis assay (COMET assay)

Single-cell gel electrophoresis or COMET assay is a technique used to determine DNA strand breaks in eukaryotic cells (Collins, 2004). Cells separated mechanically from the tissue are embedded in agarose gels on a microscope slide and lysed. Only nucleoids are thereafter left on the slides to which an electrophoresis is applied (Collins, 2004). Structures resembling comets are then observable by fluorescence microscopy and the intensity of the comet tail relative to the head reflects the number of DNA breaks (Collins, 2004).

COMET assay was performed on tissue samples collected at the end of the preliminary exposure experiment to assess DNA damage expressed as % tail DNA. To produce a baseline of DNA damage in healthy *H. panicea* cells, samples collected from the field were also included in the assay. COMET assay was performed following Hartl *et al.* (2010). Small sponge tissue samples (less than 0.5cm^3) were placed in petri dishes and immersed in Hanks Balanced Salt Solution (HBSS). The tissue samples were finely cut with a set of scalpels, separating some sponge cells from the tissue into the HBSS. The HBSS was then collected using a Pasteur pipette and filtered to remove any other structure (such as spicules). Sponge cells were thereafter embedded in agarose gels prepared onto microscopy slides, which were placed into lysing solution (2.5M NaCl, 10mM Tris, 100mM EDTA, 1% (v/v) Triton X-100 and 10% (v/v) DMSO; pH 10.0) for 24h. A minimum of 6 slides was prepared per samples. An electrophoresis was performed on the slides in a NaOH/EDTA solution (0.3 M NaOH, 1mM EDTA, pH 12): (1) slides were placed into the horizontal electrophoresis tank and covered in electrophoresis solution for 30min and (2) a 25V cm^{-1}, 300mA current was applied for 25min. The electrophoresis step took place in the dark at 4°C. Slides were washed in Tris solution (VWR) and stained with GelRed™ (VWR). Fifty randomly chosen nucleoids per slides were then examined using a Zeiss Axiophot epifluoresence microscope equipped with a Zeiss AxioCam Mrm digital camera (400X magnification; Carl Zeiss, Germany).

Statistical Analysis

A similar statistical analysis was applied to test for statistical differences in respiration rate, clearance rate and DNA damage levels between treatments and control at the end of the experiment. First the normal distribution of each datasets was checked with a Shapiro test. Bartlett tests were then used to verify the homogeneity of variances. All datasets here were normally distributed. If variances were homogenous, an analysis

of variance (ANOVA) was performed. If variances were heterogenous, a Kruskal-Wallis test was performed. This statistical analysis was conducted with RStudio (R core team, 2017).

3. Results

Physiology data

Figure B1A shows preliminary respiration data collected during a preliminary experimental run using the trial chambers (no stirring) and the Kleindienst *et al.* (2015) protocol mentioned above. Respiration rate measured during this experiment ranged from 0.14 to 5.12 µmol of O_2 per cm^3 of tissue per hour (Figure B1A), which is within the lower range reported in the literature for *H. panicea* (Osinga *et al.*, 1999). Respiration rate was very variable between individuals and this variability was greatest within the dispersed oil treatment (Figure B1A).

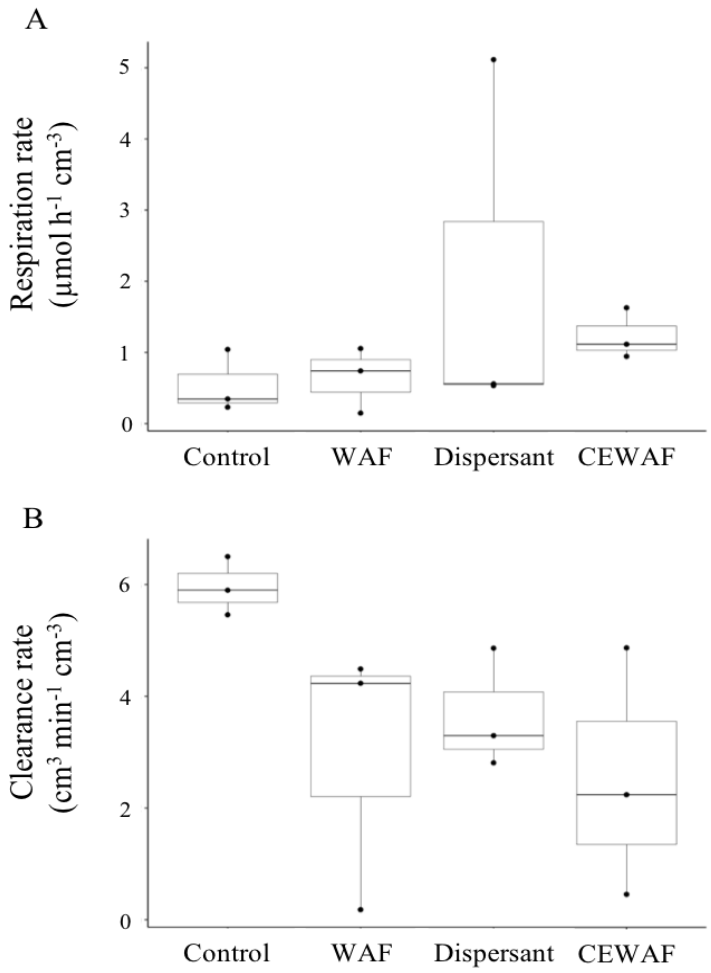

Figure B1. Preliminary physiology measurement gathered using the trial incubation chambers. (A) Respiration rate in µmol of O_2 per hour per cm^3 of tissue. (B) Clearance rate in cm^3 of seawater per minute per cm^3 of tissue. Error bars represent standard deviation.

Respiration rate seem to increase when exposed to contaminated water, especially for the dispersant and dispersed oil treatments (Figure B1A). However, due to high variability between individual sponges and the low number of replicates, no statistical differences were detected (Kruskal-Wallis test, χ^2=3.3077 and *p*-value= 0.3466).

Figure B1B shows preliminary clearance data collected during the preliminary experimental run using the trial chambers and the Kleindienst *et al.* protocol (2015) mentioned earlier. Clearance rate measured during this experiment ranged from 0.18 to 6.50 cm^3 of water per cm^{-3} of tissue per min (Figure B1B), which is within the lower range reported in the literature for encrusting sponges (De Goeij *et al.*, 2008ab) . Additional stress caused by moving the sponges into physiology chambers one hour prior to the respiration and clearance rates measurements could explain the low clearance rates measured. Individuals exposed to contaminated seawater displayed lower clearance rates with higher variability compared to the control treatment (Figure B1B). Overall clearance rate seemed to decrease when sponges were exposed to contaminated seawater, but no significant differences could be detected (ANOVA, F-vale=2.2942 and *p*-value= 0.1547).

COMET Assay

COMET assay was trialled on samples from the preliminary experiment conducted with the Kleindienst *et al.* protocol (2015). Nucleoids were observed on the slides and could be scored accordingly (Figure B2AB). On average, baseline samples displayed 5.6% tail DNA and control samples displayed 4.2% tail DNA, which is higher than what has been observed for other organisms such as mussels (Hartl *et al.*, 2010) but in accordance with the only available sponge study (Akpiri *et al.*, 2017) (Figure B2C).

Sponge cells and in particular pumping cells or choanocytes display the shortest cell cycle, observed in multicellular organisms (De Goeij *et al.*, 2009). These cells can be expelled from the sponges and replaced by new choanocytes every six hours. Heightened basal DNA damage could be a consequence of the shorter cellular cycle for some of the sponge cells. Average scores for samples from treatment conditions ranged from 10.7 to 13.0% tail DNA. Overall % tail DNA variability was very high between nucleoids measurements across all conditions including baseline, controls and treatments. ANOVA analysis on COMET data revealed statistical differences between the three treatment conditions (WAF, CEWAF and dispersant) compared with baseline and control data (F-value=28.37, *p*-value < 2.2e-16). DNA damage in sponges after exposure to

benzo[a]pyrene (BaP) and BaP photo-derivatives has, however, been previously observed in sponge *Tethya lyncurium* (Zahn *et al.*, 1981, 1983).

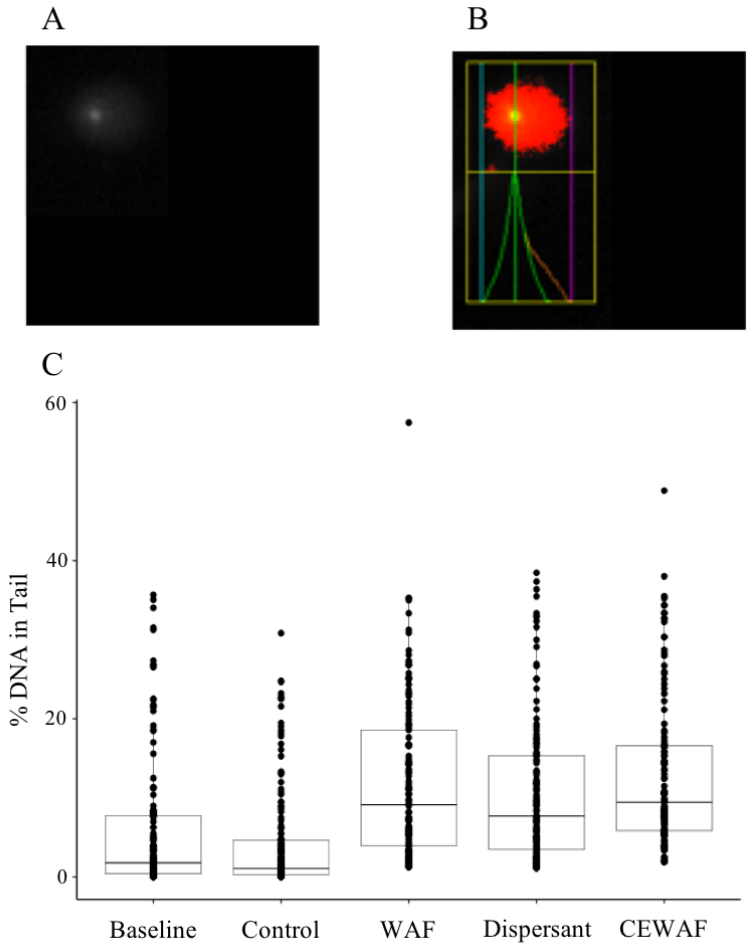

Figure B2. Single-cell gel electrophoresis (or COMET) assay analysis. (A) Example of a COMET (B) Example of an analysed COMET (C) Results of COMET analysis.

4. Conclusion

The preliminary results presented in this appendix suggest that (1) respiration rate could be monitored to determine the survival of *H. panicea* to an exposure (2) clearance rate could be used to identify sub-lethal effect of an exposure (3) molecular effects can be expected from the COMET assay results. High variation in % tail DNA even within the baseline samples are however still observable in this data and several attempts were made to produce a less variable baseline without success. Furthermore, samples for COMET assay need to be processed as soon as possible after collection, which was not always possible when physiology measurements were also needed. COMET assay was therefore not pursued in further experimental work.

Appendix C

Experimental Work Conducted on *Myxilla sp*

1. Overview

This appendix presents preliminary work conducted on *Myxilla sp,* collected at 10m depth by SCUBA diving in Loch Creran (thanks to the Heriot-Watt Dive Team). *Myxilla sp* used in this work is a bright orange encrusting sponge colonising scallop shells. Upon collection, the shelves were brought back to Heriot-Watt University and kept in retention tanks before being used in the experiments (as described in appendix B).

Two main objectives of the work were (1) to determine if results obtained with *Myxilla sp* are in accordance with the *Halichondria panicea* findings (2) to determine if the protocols used to prepare water-accommodated oil fraction (WAF) can impact the results of exposure experiments. Two experiments were therefore designed. First a dose-response experiment was conducted using the chemical response to oil spills ecological research forum (CROSERF) protocol (Aurand and Coehlo, 2005) and physiological results were compared to the results from the *H. panicea* experiment presented in Chapter 3. Second a dose-response experiment with the Kleindienst *et al.* protocol (Kleindienst, *et al.,* 2015) was conducted and results between the two *Myxilla sp* experiments were compared.

2. Experiment 1: comparison across species

Material and methods

Protocols defined in chapter 3 were followed for this experiment. A range of WAF solutions were produced following the CROSERF protocol with crude oil loadings varying from 0.01 to 5g/L. Sponges were exposed for 48h before respiration and clearance rates were measured. Water samples from this experiment were collected and measurements of polyaromatic hydrocarbons (PAHs) concentration occurred as described in chapter 3 to allow comparison with the second experiment discussed in this appendix.

Dose-response model with a Weibull 1 three parameters (upper asymptotic limit, slope and ED50) function were applied to physiological data sets which showed dose-response trends. As the aim of this study was to compare the results between two sponge species, independent model estimates for each sponge were calculated and then compared. A lack-of-fit test was then carried out to determine the model significance and

comparison tests between estimates were finally computed. All statistical analysis was done on Rstudio with the package drc (Ritz *et al.,* 2015; R Core Team, 2017).

Results and Discussion

Respiration rate measured in *Myxilla sp* varied between 0.18 and 6.13 µmol hour^{-1} cm^{-3} (Figure B-1A). This is in accordance with respiration rate available for encrusting sponge in the literature (Osinga *et al.,* 1999). *Myxilla sp* respiration rate measured here were also in accordance with the range measured in *H. panicea* (data from chapter 4), albeit overall lower (Figure B-1A).

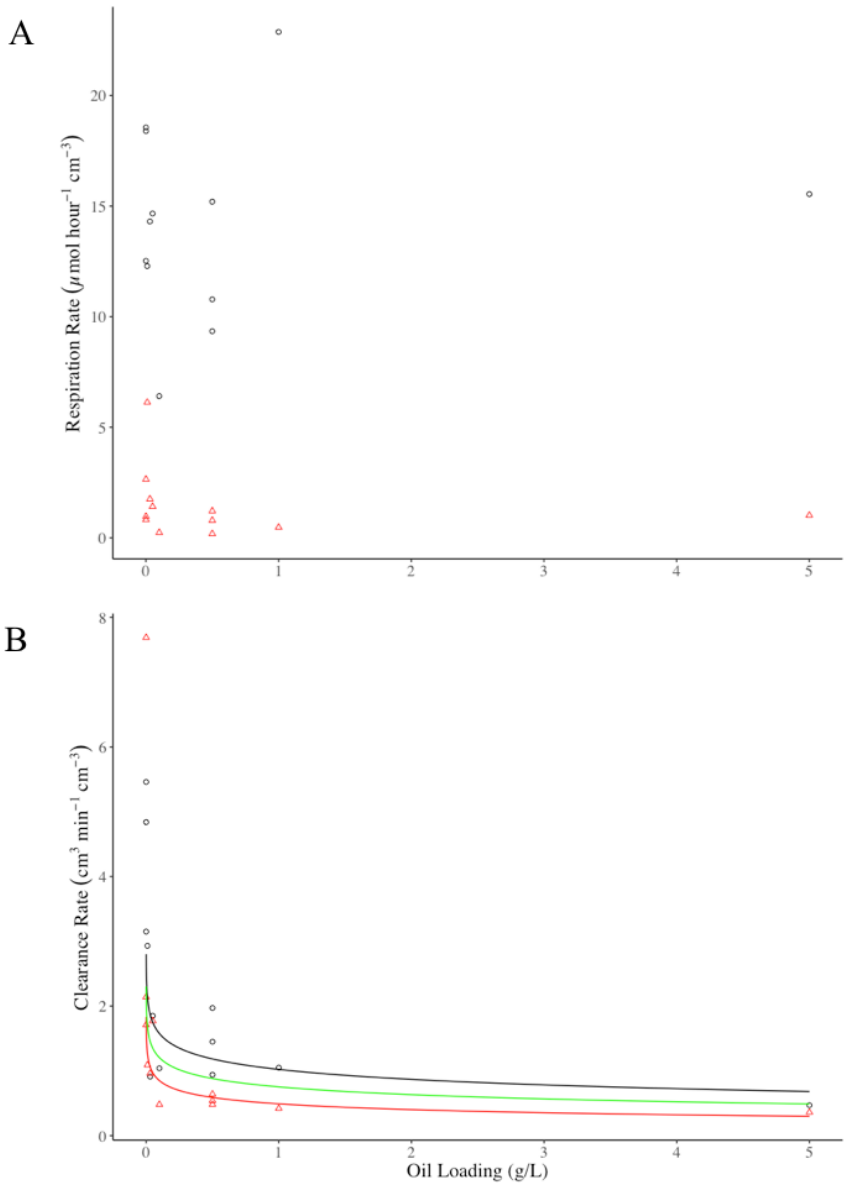

Figure B-1. Comparison between physiology measurements gathered on *Myxilla sp* (red triangles) and *Halichondria panicea* (black dots) (A) Respiration rate (µmol hour^{-1} cm^{-3}) at increasing oil volumes (B) Clearance rate (cm^3 min^{-1} cm^{-3}) at increasing oil volume. The *H. panicea* model estimated trend is plotted in black curve. The *Myxilla sp* model estimated trend is plotted in red. The common model estimated trend is plotted in green.

No dose response relationship between respiration rate and oil loading could be detected here and no dose response model was therefore fitted to the data.

Myxilla sp clearance rate measured in this study ranged from 0.36 to 6.12 cm^3 min^{-1} cm^{-3} (Figure B-1B). This is in accordance with values available in the literature for other encrusting sponges (De Goeij *et al.,* 2008ab) and with values measured in *H. panicea* (data from chapter 4). Clearance rate in *Myxilla sp* decreased with increasing oil loading as observed for *H. panicea* (Figure B-1B). A dose-response model with independent estimates for the two species was successfully fitted to the data (lack-of-fit test; F-value= 0.09; *p*-value= 0.99). Statistical comparisons between estimates (upper asymptote, ED50 and slope)) of the two sponge species were then performed and no statistical difference was detected. Data from the two species can therefore be modelled in one single curve (green curve, Figure B-1B).

Physiology results from this experiment provide evidence to show that the response seen in *H. panicea* is similar to what can be observed in other sponge species. Further molecular work on *Myxilla sp* tissue samples are required to fully verify the *H. panicea* results.

3. Experiment 2: comparison across WAF protocols

Material and methods

Protocols defined in chapter 3 were followed for this experiment. A range of WAF solutions were produced following the Kleindienst *et al.* protocol (2005) by applying dilution/concentration factors varying from 0.3 to 3 to the preparation of working WAF solutions. Sponges were exposed for 48h before respiration and clearance rates were measured. Water samples from this experiment were collected and measurements of PAHs concentration occurred as described in chapter 3 to allow comparison with the first experiment discussed in this appendix.

Physiology measurements were plotted as a function of phenanthrene concentration, present ubiquitously in the water samples. Dose-response model with a Weibull 1 three parameters (upper asymptotic limit, slope and ED50) function was applied to physiological data sets which showed dose-response trends. As the aim of this study was to compare the results between the two protocols, independent estimates for each protocol were calculated. A lack-of-fit test was then carried out to determine the model significance and comparison tests between estimates were finally computed. All

statistical analysis was done on Rstudio with the package drc (Ritz *et al.,* 2015; R Core Team, 2017).

Results and Discussion

Two to four ring PAHs were often detected in the water samples from either protocols where as more complex PAHs appeared to be rare in the water samples. This is in accordance with the results from chapter 4. Overall concentration varied between 0.014 to 2.70 µg/L. High levels of variation (large error bars) is due to the large range of oil loadings/dilution factors tested here. Variation is highest in the Kleindienst samples due to the smaller number of samples analysed (n=6 against n=12). Overall sponges were exposed to a similar range of concentrations in both experiments.

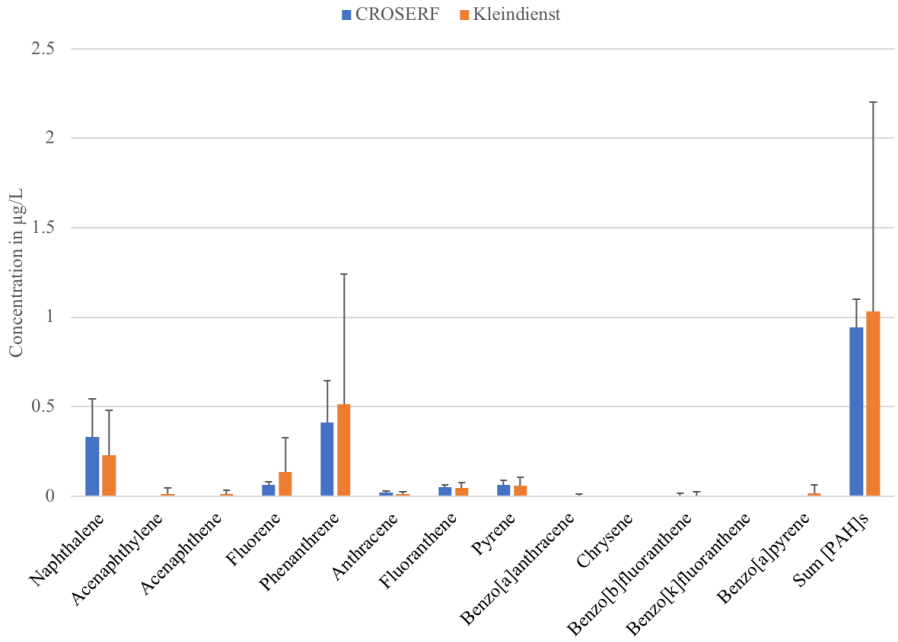

Figure B-2. Average concentrations of 13 individual polyaromatic hydrocarbons (PAHs) and average total concentration of PAHs in CROSERF and Kleindienst water samples from *Myxilla sp* experiments.

Myxilla sp respiration rate measured in the Kleindienst experiment varied between 0.32 and 8.15 µmol hour^{-1} cm^{-3} (Figure B-3A). This is in accordance with respiration rate measured for encrusting sponge in the literature (Osinga *et al.,* 1999). *Myxilla sp* respiration rate measured here were also in accordance with the range measured in the CROSERF experiment (see previous section) (Figure B-3A). No dose response relationship between respiration rate and oil loading could be detected here and no dose response model was therefore fitted to the data.

Myxilla sp clearance rate measured in the Kleindienst experiment ranged from 0.29 to 7.92 cm^3 min^{-1} cm^{-3} (Figure B-3B). This is in accordance with values available in the literature for other encrusting sponges (De Goeij *et al.,* 2008ab) and with values measured in the CROSERF experiment (see previous section). Clearance rate in *Myxilla sp* decreased with increasing oil loading as observed for the CROSERF experiment (Figure B-3B).

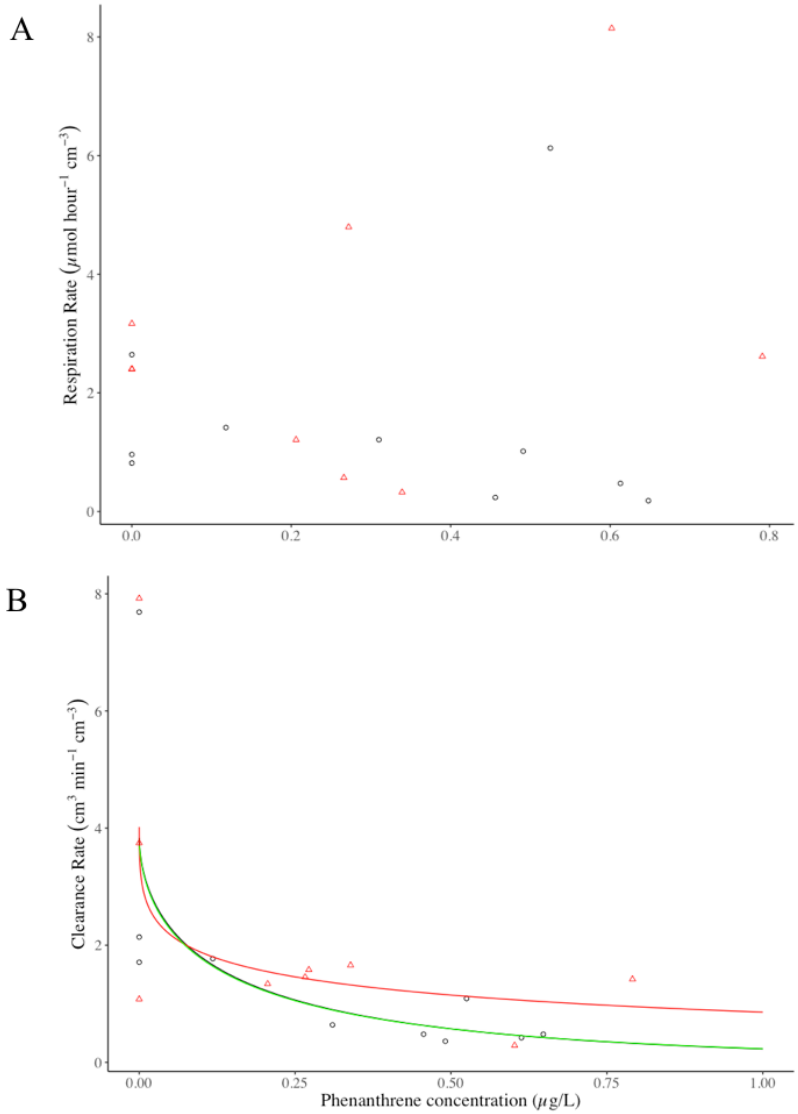

Figure B-3. Physiology measurements on *Myxilla sp* exposed to water accommodated oil fractions prepared with the CROSERF (Aurand and Coelho, 2005) protocol (black dots) and Kleindienst *et al.* (2015) protocol (red triangles). (A) Respiration rate (µmol $hour^{-1}$ cm^{-3}) at increasing phenanthrene concentrations (B) Clearance rate (cm^3 min^{-1} cm^{-3}) at increasing phenanthrene concentrations. The CROSERF model estimated trend is plotted in black. The Kleindienst model estimated trend is plotted in red. The common model estimated trend is plotted in green.

A dose-response model with independent estimates for the two species was successfully fitted to the data (lack-of-fit test; F-value= 0.014; *p*-value= 1.0). Statistical comparisons between estimates (upper asymptote, ED50 and slope) from the two protocols were then performed and no statistical difference was detected. Data from the experiment can therefore be modelled in one single curve (green curve, Figure B-3B).

Physiology results from this experiment provide evidence that protocols for the production of WAF do not seem to impact the results of sponge exposure experiments. Further molecular work on *Myxilla sp* tissue samples could be conducted to fully verify the physiology results.

Appendix D

Field and Experimental Work Conducted Offshore Nuuk, Greenland

1. Overview

In the summer 2016, a series of diving surveys around Nuuk were organised by Dr K. Schoenrock (U. of Glasgow, now NUI Galway) and Dr N. Kamenos, in collaboration with the Calving Glaciers: Long Term Validation and Evidence research project (CALVE Leverhulme Trust Research project Grant 2014-092). The diving component initially aimed at better understanding the shallow-water coastal Greenlandic environment and determining the biodiversity associated with kelp forest and maerl bed habitats (see appendix A for publication). Thanks to the collaboration with Dr. K. Schoenrock and Dr. N. Kamenos, it was possible to also sample for sponges. The objective of the sponge sampling was to:

(1) Identify sponge species present in shallow-water offshore Nuuk

(2) Conduct exposure experiments on sponges collected offshore Nuuk

Greenland is believed to possess important offshore oil reserve (Harsem *et al.*, 2011) which are currently being explored and assessed (Hurup Olsen and Hansen, 2014). Providing a baseline for the presence and resilience of important marine organisms such as sponges is therefore of great importance.

2. Sponge identification

Sponge samples were collected during each dive undertaken. Dives were performed on both kelp forests and maerl beds. Most sponges were found to be associated with the kelp stipes although two samples were found on maerl beds. A set of 12 sponge species was sampled during the fieldwork and identification is currently underway, in collaboration with sponge taxonomist Dr. C. Goodwin. Figure C-1 shows examples of sponge morphotype, skeleton, and spicule types found during the identification process. Table C-1 provides a summary of the progress made so far.

3. Experimental work

Experimental work on two different sponge species (sponge species 5 and 7) was conducted while in Nuuk. For each experiment, twelve sponge individuals were collected and brought back to the Greenland Institute of Natural Resources (GINR). Once at the GINR, samples were placed in incubation chambers and left to recover for 4 days prior to the exposure. Simple incubation chambers were used in these experiments: glass jars of 750 mL of volume were equipped with inflow and outflow glass tubes through a metal lid. Peristaltic pumps were used to circulate water through the chambers at a rate of one

litre per day to ensure a good water mixing. After recovery, a 48 hour exposure to water accommodated oil fraction (WAF), chemically enhanced WAF (CEWAF) and dispersant contaminated seawater was conducted. After the exposure, samples were kept in their chamber for another 48 hours before the end of the experiment. The experimental apparatus was kept in a GINR cold-room, set to 2° C.

Respiration rate and clearance were measured before, during and after the exposure. Unfortunately, water samples for the determination of clearance rate were lost during the transport back to the UK and so only respiration rate is available. In accordance with the findings of this thesis, respiration rate was highly variable and did not seem impacted by the treatment. Tissue samples were also collected before, during and after exposure. Unfortunately, primers from the literature (Webster *et al.,* 2013) and developed during the PhD project do not seem to produce any amplicon on the Greenlandic species. Funding to allow transcriptomic sequencing of RNA extractions is therefore being sought for.

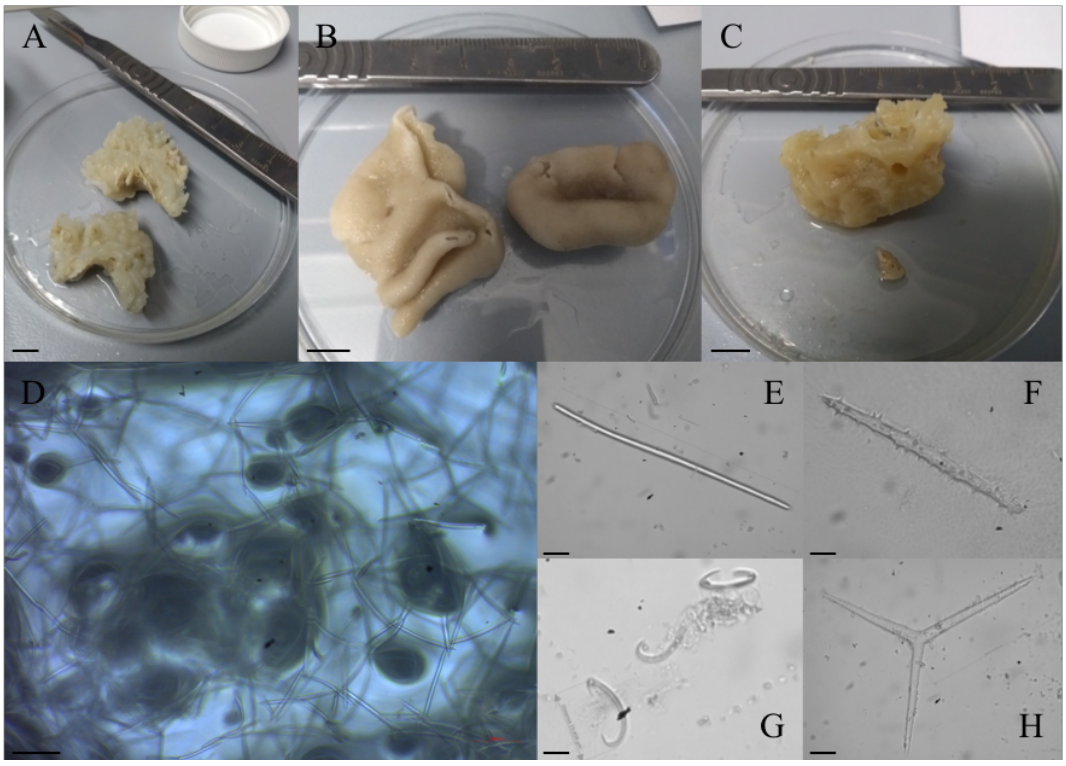

Figure C-1. Sponge sample collected in Greenland. (A) Sponge with papillae (sponge sample 2). (B) Lobose sponge (sponge sample 3). (C) Cushion sponge (sponge sample 4). (D) Isodictyal reticulation skeleton found in sponge 12. (E) Subtylostyle from sponge sample 5. (F) Acanthostyle from sponge sample 4. (G) Sigmas from sponge sample 10. (H) Triactine from Calcarea sponge sample 3. Scale bars: (A) to (C) 1cm, (D) 100 µm, (E) and (H) 20 µm, (F) and (G) 5 µm.

Table C-1. Sponge sample identification data. Skeleton organisation, spicule type and size class and potential identification is given. "?" means that the identification to the genus level is still in progress.

Sponge sample	Skeleton	Spicule type	Size range	Class	Potential Identification (C. Goodwin)
Sponge 1	Still undefined	Acanthostyle	123-170	Demospongiae	*Myxilla sp*
		Sigmas	12-57		
		Isopalmate Chelae	23-31		
		Tylote	185-230		
Sponge 2	Isodictyal reticulation	Oxea	225-490	Demospongiae	*Halichondria (Eumastia) sitiens*
Sponge 3	Still undefined	Triactine	65-175	Calcarea	
		Diactine	115-420		
Sponge 4	Alveolate skeleton	Acanthostyles	102-235	Demospongiea	*Myxilla sp*
		Acanthostyles	100-125		
		Sigmas	25-30		
		Tylote	130-170		
Sponge 5	alveolate skeleton	Oxea	120-225	Demospongiae	*Halichondria sp*
		Subtylostyle	160-185		
Sponge 6	Isodictyal reticulation	Oxea	75-100	Demospongiae	*Haliclona sp*
		Oxea	60-70		
Sponge 7	Anisotropic	Oxea	50-110	Demospongiae	*Haliclona sp*
Sponge 8	Still undefined	Diactine	230-270	Calcarea	
Sponge 9	Still undefined	Acanthostyle	50-125	Demospongiae	*Myxilla sp?*
		Isopalmate chelae	75-85		
		Tylote	220-850		
Sponge 10	Still undefined	Sigmas	20-60	Demospongiae	*Myxilla sp?*
		Acanthostyle	110-200		
		Tylote	170-250		
		Chelae	15-20		
Sponge 11	Still undefined	Fusiform oxea	100-235	Demospongiae	*Halichondria sp?*
Sponge 12	Isodictyal reticulation	Fusiform oxea	100-120	Demospongiae	*Haliclona sp?*
		Oxea	100-120		

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